User's Manual

MicroVigene™

Version 2.99

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VigeneTech

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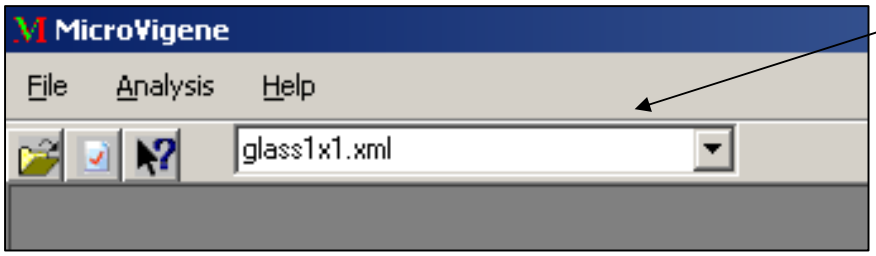
I QUICK START OF MicroVigene™

1. Starting MicroVigeneTM

To launch MicroVigene™:
Select **Start|All Programs|MicroVigene** (The "***Start***" icon that is located on the lower left corner of your computer screen) **or** double click

on the screen.

Main Menu is displayed when no image is loaded. It looks like this:



Template drop down box

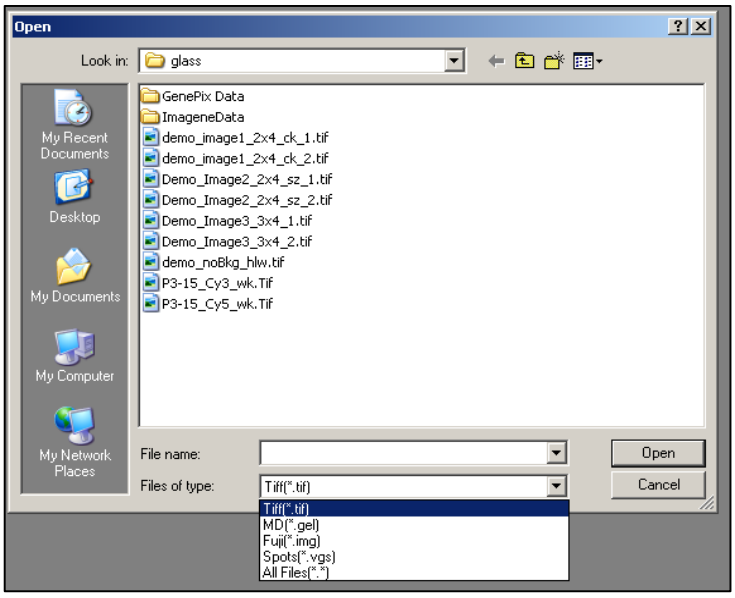
Select the right template for your image from the dropdown list.

Note MicroVigene™ will automatically retrieve the last settings used as the default. The template file selected in the template drop down box will be the template that was used last time.

2. Analyzing Array Image with Pre-defined Template

Step 1: Open image file

Click the on the Toolbar to show the following window:



MicroVigene™ supports **.tif**, **.gel** and **.img**. See more information in the Image Section.

Note: MicroVigene™ works better with a 16-bit format image files.

Step 2: Select the template file

Template file, in .xml format, has to be copied in the template folder before you launch MicroVigene™. Then you can find the template file in the template dropdown list.

You can instruct the template files to be stored at any folder. The default folder is *C:\Program Files\VigeneTech\MicroVigene\Template*.

Once the image is loaded onto MicroVigene™, the **Application Menu** will be in display. It looks like this:

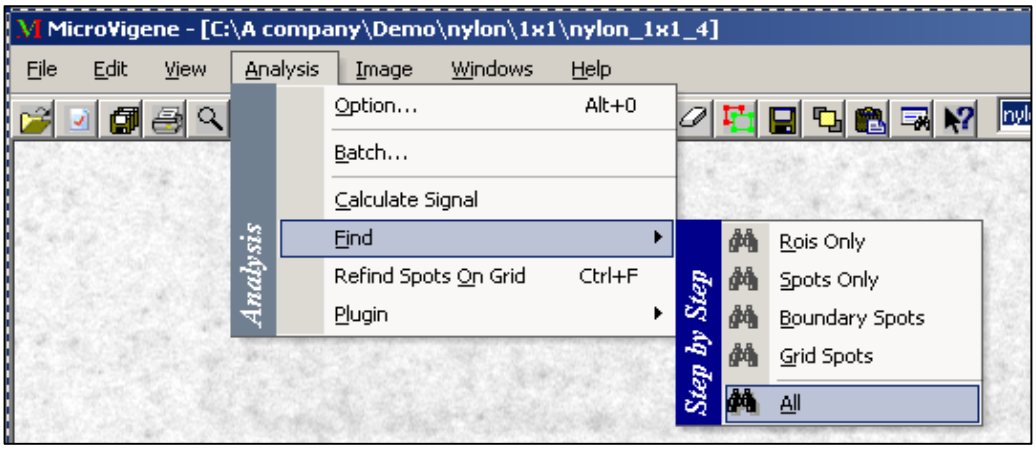
Step 3: Analyze image

When the correct template is selected, one mouse click is all you need for your image analysis.

Click the green **GO** (find all) button on the Toolbar in the Application Menu,

or

select **All** from dropdown list, **Analysis|Find|All** as shown in the following::



MicroVigene™ will automatically find the ROI (Region Of Interest), place the grid, find the spots, subtract the background, remove the dust, and quantify spots.

Step 4: Save Image Analysis Results

Output Data File:
MicroVigene™ image analysis output data can be saved as **.txt** and **.xml** files in the same folder as its image file folder. Any other formats can be supported via plug-ins.

Grid and Spots File, .vgs:
MicroVigene™ spot files are saved as **.vgs** format. This is a VigeneTech proprietary grid and spot data format. Grid, spots, main configuration setting, and manually defined ROI frames can be saved. It also is saved in the same folder as its image file. A vgs file can be loaded with its original image file under the same name as .tif or .gel extension that is in the same folder as its image file.

Save:

Click the button on the Toolbar; both text (**.txt**) and spots (**.vgs**) will be saved.

Step 5: Print out the image overlaid with grid and spots

Click the button on the Toolbar to print the image that has both grid and spot images.

II DATA QUALITY

1. Viewing Image Analysis Results

After one mouse click analyzes the image, the screen will show the red ROI outline, yellow grid line, and cyan spot's boundary. The good spots and strong spots will show the exactly the signal segmentation, others will just show a circle with its center as the mass center of the spot.

View Spot Boundary in One Color:
As default, the spot boundary will show in cyan color. The good spots and strong spots will show the exactly the signal segmentation, others will just show a circle with its center as the mass center of the spot. It can be defined in the section of Options::Basic

View Spot Boundary in Multiple Color:
The image also can show different colors of the spot boundary. Based on the measurement of the spot, as desecrated in Section of Spot Info, there are cyan, green, purple and red spot boundary respectively to the quality measurements.

2. Grid and Spots Quality

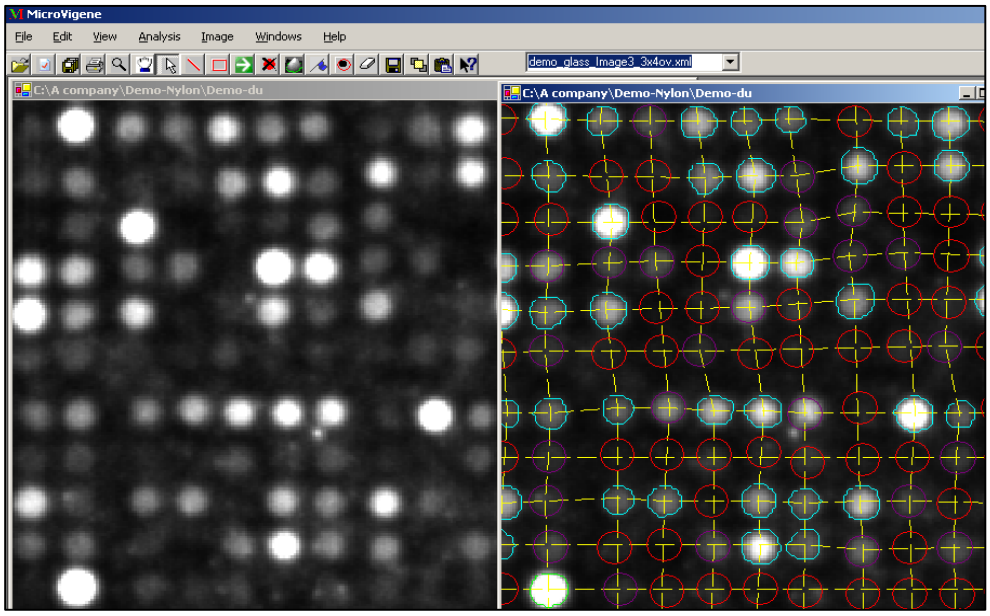
MicroVigene™ can automatically place flexible grids and perform accurate signal background segmentation. The benefits of accurate segmentation are:

high accuracy,

high sensitivity, and

100% data reproducibility

The following screen capture illustrates how well MicroVigene™ can find the actual spot boundary with a flexible grid:



3. Visualizing Tools

A set of tools is available to visualize the quality of image analysis as list below.

Select or Highlight Spots, ROI, Slide:

Single Object

Like spots, ROI, slide, sides of ROI, and anchor points are objects. Click the button on the Toolbar, then move the arrow cursor to the object, and click to select.

Grouping Objects or Area (rubber band)
Move the arrow cursor to the top left corner of an area, click and hold down the mouse button to drag over the area. The cursor-crossed area or objects will be selected.

Moving and Scrolling Images

Click the button on the Toolbar. Move the hand cursor to the image area, holding down the left mouse button to move the image in any direction that the cursor directs.

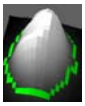
Image Zooming in/out

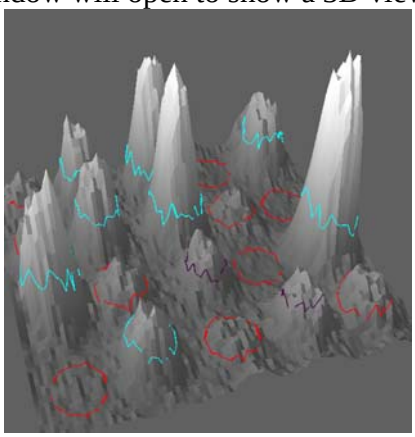
Double zooming in or half zooming out:

Click the button on Toolbar, then move the cross cursor onto the image. Left click to zoom in (enlarge) in double size, or right click to zoom out (decrease) in half size.

Zooming in for selected area (rubber band):
Move the cursor to the interested area. Click on the top left corner of the area, then click and hold down the left mouse button to drag over the area. The cursor-crossed area will be enlarged to fill up the entire view window.

3D Spot View

Click the  button on the Toolbar, and move the "+" cursor to the image area. Hold down the left mouse button and drag it over the interested area to view its 3D profile of the selected spots. A new window will open to show a 3D view.



Note: The 3D view function uses a lot of memory. Therefore, if large area is selected, it will take long time for the 3D view window to appear.

Spot 2D Magnifier

Closely look up spots in the 2D view by selecting the menu dropdown list, **Application Menu::Image|Magnifier** to open a **Zoom** window.

Magnifying the Area:
Move the cursor into the **Zoom** window. Click and drag out to the area in the image window for a closer look. The Magnifier window will view the area as the mouse moves.

Zooming in and zooming out:
Move the scrolling bar on the right side of the Zoom window to increase the magnifier and move up to decrease the magnifier.

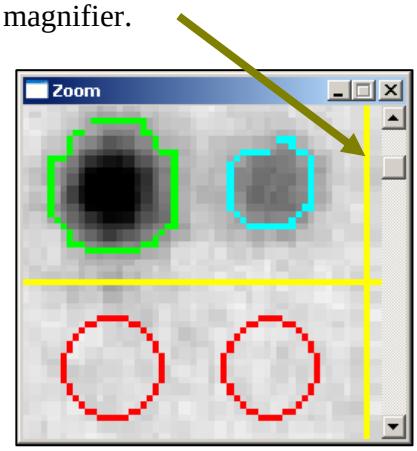


Image in original resolution View

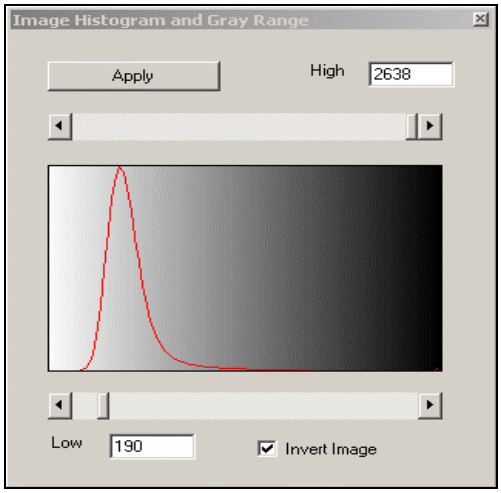
Select the menu dropdown list, **Application Menu::Image|Default,** to zoom the image to its standard size.

Image Full Screen View

Select the menu dropdown list, **Application Menu::Image|Zoom to Fit** to view the whole image.

Image Range and Inverting an Image

Select the menu dropdown list, **Application Menu::Image|Range** to open the **Image Histogram and Gray Range** box:



Invert Image:
Check the **Invert Image** check box to invert or un-invert the image

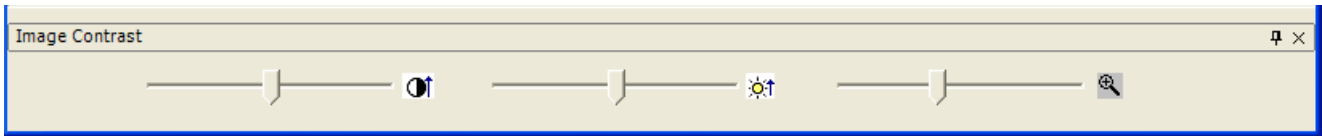
Adjust Image Gray Level Range:
Move the scales on the scroll bar to adjust high and low range of pixel gray. **High** and **Low** text boxes are the high and low cut-off gray values.

Click on the **Apply** button (as shown above) to finalize your choice.

Note: Inverting and adjusting image visualization will not change any pixel value in the image.

Image Contrast, Brightness, Zoom in/out:

Select the menu dropdown list, **Application Menu::Image|Contrast** to open the **Image Image Contrast** panel at the bottom on the screen.



Move the scroll bar of , and to adjust contrast, brightness and zoom in/out respectively. The changes will instantly appear on the image.

Click to close the panel.

III SPOT INFO

1. Quality Determination Criteria

There are color -coded spot boundaries used to differentiate the spot quality. It can be chosen to show up on the screen, see Configuration Section:

All spots on the array image are categorized and color-coded according to its quality:

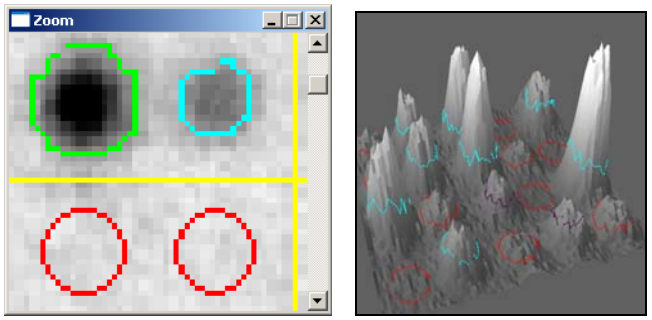
Reliable spot color code: **cyan**, **green**, and some **purple**

Un-reliable spots color code: **red**, **purple**

Reliable and un-reliable spot number codes are in the output data file (the **.txt** file):

Good: cyan = (1)
Blooming: green = (0)
Poor: purple = (3)
Weak: red = (5)
Flag: "+" in the center of spots = (6)
Spot Dust: Blue in red spots (non-zero value of Vol_dust)
Background Dust: Yellow boundary.

For Cyan (1) and green (0) spots, MicroVigene™ shows true spot boundaries in 2D and 3D view. For purple (3) spots, MicroVigene™ shows true boundaries in 3D and 2D circle . Red (5) spots show a circle centered with its mass center in both 2D and 3D view window.



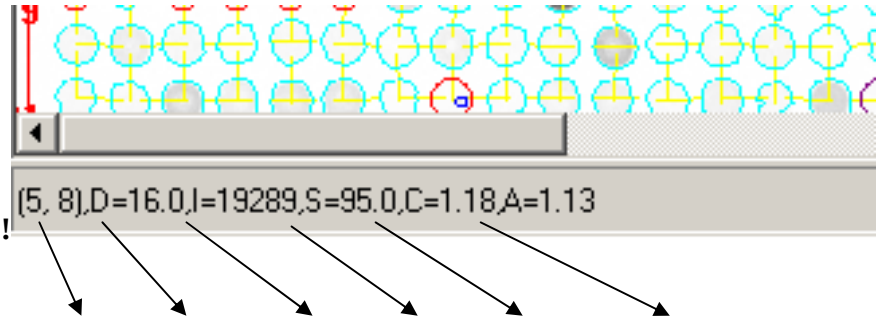
Note: Spot type is determined based on the definition in the template file. There are five parameters in the dialog box for you to define which will affect the outcome of spot quality, i.e., the number of reliable spots you will get from an image.

2. Spot and Pixel Information on Status Bar

Click the button on the Toolbar, move the cursor to the image and highlight the spot to view:

Left Status Bar

View spot information as shown below:



Error! (row, column), **D**iameter, **I**ntensity, **S**olidity, **C**ircularity, **A**spect ratio

Note: If a Platemap file is loaded, the gene ID will show in this status bar as well.

Right Status Bar

View pixel information: x, y, z.

Move the mouse cursor (arrow) on the image to read the right bottom Status Bar for pixels. X and y values indicate the cursor location measured by pixels; the z value indicates the gray level for non-color images (signal intensity) or color levels (for color images).

Positive and Negative spots images

Move the cursor into the spot areas and at the background areas, compare the "z" value between spot and background pixels to determine the image as:

Positive spot: the spot "z" value > background "z" value

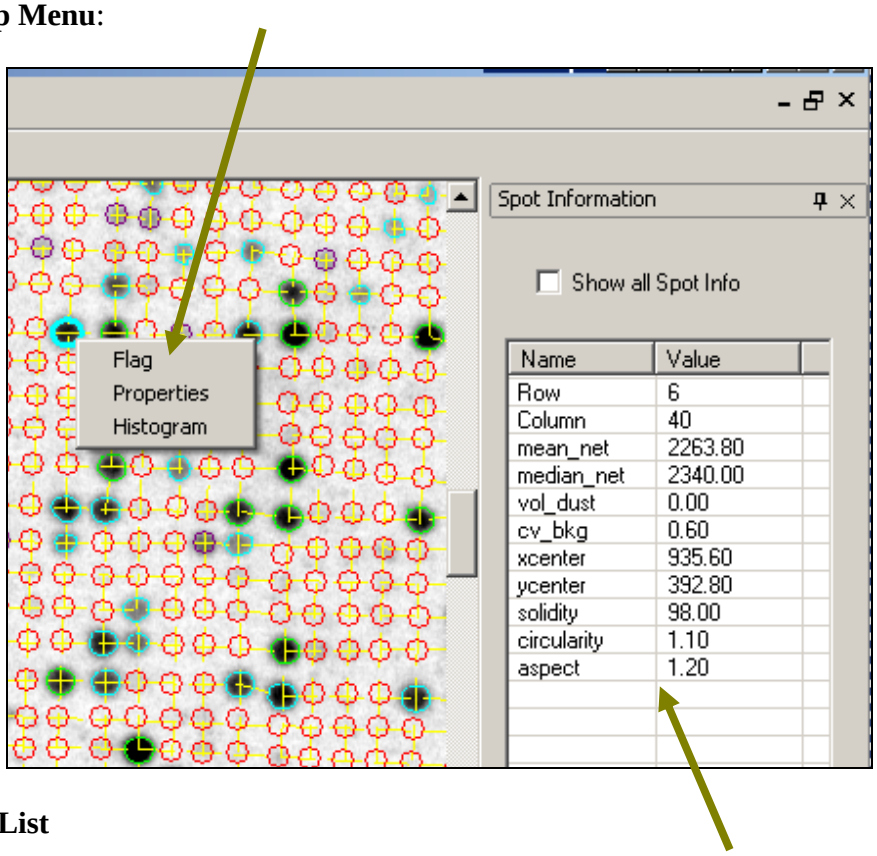
Negative spot: the spot "z" value < background "z" value

Note: This information is important in order for you to set-up a template file.

3. Single Spot Info

Spot Pop-up Menu

Click the button on the Toolbar, move the cursor to the image to highlight a spot. Right click to show the **Spot Pop-up Menu**:



Spot Info List

Select the **Spot Pop-up Menu::Properties** to open the **Spot Information** list.

Right panel shows all the measurements for this spot in the output .txt file.

Check the **Show all Spot Info** check box to list all 24 measurements for all of these spots.

View Spots Histogram

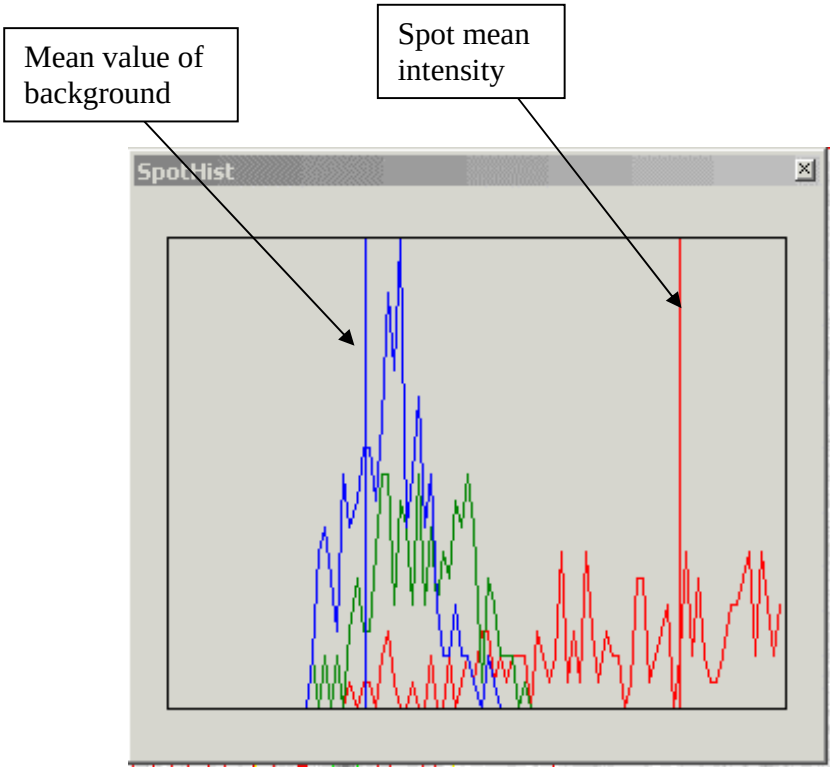
Select the **Spots Pop-up Menu:: Histogram** to open Histogram dialog box.

The red distribution line is the pixels from the spot area, the green is from the buffer zone, and the blue is from the background. The vertical red line represents the spot mean intensity, and the vertical blue line represents the mean value of the background.

Red---spot

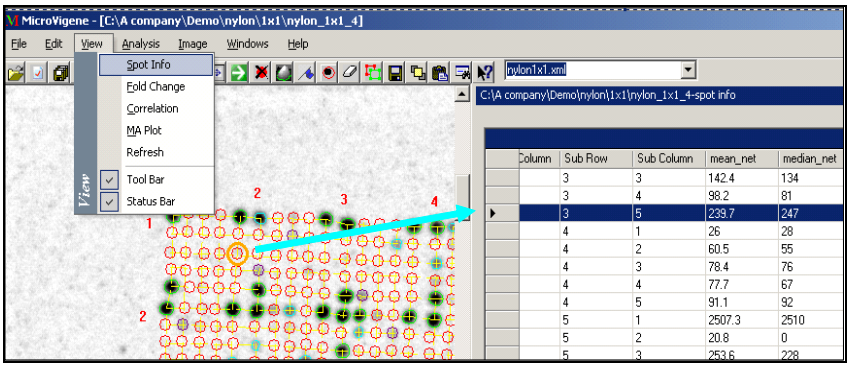
Green---Buffer Zone

Blue---Background



4. View All Spot Info

Select the menu dropdown list, **Application Menu::View|Spot Info,** as shown in the following screen capture. A Spot Info list will be shown on the right panel. All spots information can be viewed.



Spot Info List Items:
This list shows the same layout as the output file. The listed measurements are specified in the Options dialog box, under the Output tab.

Sort Spot Info list
This list can be sorted as a spreadsheet by selecting any variables. Simply click on the column head at the top.

Use this feature to check how many dusty spots are found by sorting the spot based on **Vol_dust**, and how many blooming spots are found by sorting the spot based on **Type** value of 0, etc.

Spot Image and Info List Association
For each spot in the list, click on the far left gray column (as shown in above screen capture in blue arrow); the corresponding spot with a large orange circle around it will be shown on the image. Click on the spot; the corresponding row of spot information on the list will be highlighted as well.

Close Spot Info List:
Click on “x” at the top right corner of the list panel to close the Spot Info list.

IV SPOT DATA ANALYSIS

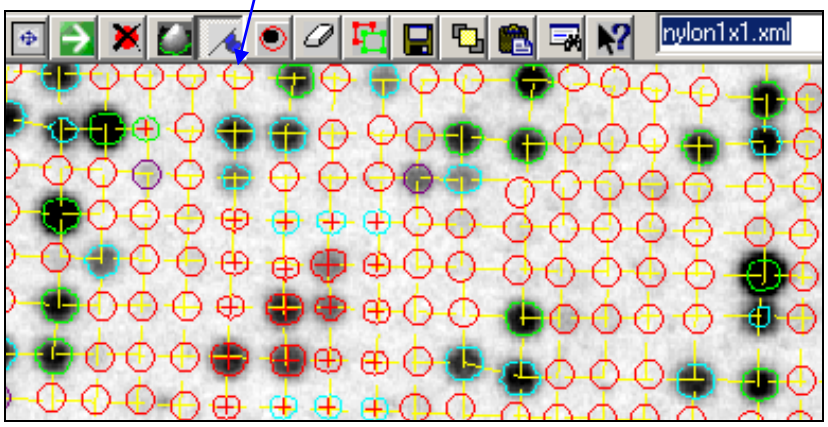
MicroVigene™ has implemented automatic and manual functions to flag spots, find and remove dust.

1. Flag Spots

Manually Flag Spots

Flagging a Single spot

Left mouse click on the button on the Toolbar and move the circle cursor to the spot, then click to flag this spot. The red cross will show up in the center of the flagged spot.



You could also use the **Spot Pop-up Menu**::**Flag** to flag a selected spot.

Flag group of spots

Click on the button, and move the cursor to the image. Then hold down the left mouse button and drag it over the area where a group of spots need to be flagged. Release the left mouse button. All spots in the area will be flagged with a red- cross at the center of each spot. It is also called rubber-band spots to flag a group of the spots.

Un-Flag the Spots

Move the flag spots circle cursor on the flagged spots, right mouse click to un-flag the spots. Hold down the left mouse button and drag cross the area to un-flag all flagged spots in that particular region.

Auto Flag Spots

Auto Flag Spot with Pre-defined Spot Quality Thresholds

MicroVigene™ can automatically detect the spot are exceed the threshold for the quality measurement, such as minimum spot intensity and spot shapes and background. Select flag options in **Flag Mode** drop down list and with set up at Min signal (std) **Options** dialog box, under **Grid** tab. See Configuration and Template section.

Auto Flag Contaminated and Dusty Spots

MicroVigene™ can also automatically detect highly contaminated and dusty spots, when **Min. Mean** value set is to negative in **Options** dialog box, under **Spot** tab and **Dust** section. See configuration section.

Control Type Flag

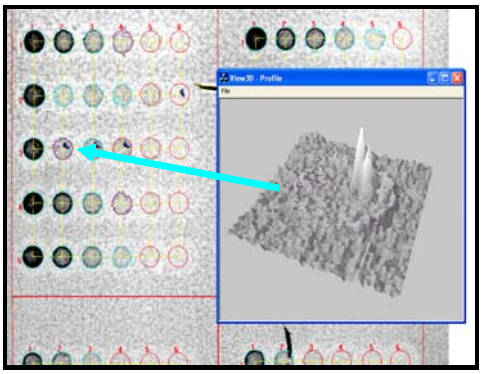
MicroVigene™ can take the input from the platemap file with the control column to define the flag spots and automatically assign those spot types to the value of 6. This can be supported by a customized plug-in program.

2. Finding and Removing Dust

Manually Finding Dust

Dust in single spot

Click on the Toolbar and move the cursor to the spot and click. If there is dust, it will find the dust or it will dust in the spot and exceed the threshold. The blue contours show the areas where dust was found. See configuration, Spot section for the dust threshold setting.



Dusts in a group of spots:

Click on the Toolbar and move the cursor to the image while holding down the left mouse button and drag it over the area of interest and release the mouse (Rubber band function to select area of interest). The blue contour will show the spots with dust found in the area.

Manual dust Removal

After manually finding the dust (or undo dust find), select the menu dropdown list, **Application Menu::Analysis|Calculate Signal,** to remove the dust from the spot intensity measurements.

Note: The intensity of dust can be viewed by selecting the dusty spot, then click the right mouse button. Select **Spots Pop-up Menu::Properties.** And then click **View all Spot Info** check box to see **Vol_dust** value.

Manual Un-do dust find and dust removal

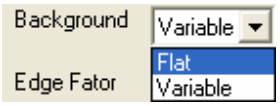
Click on the Toolbar, then click on the spot to remove the blue dust contour. **Vol_dust** will be zero. Use the "Rubber band" function to erase dust in multiple spots in the area.

Automatic dust find and dust removal

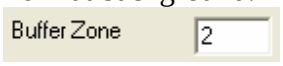
MicroVigene™ automatically detects and removes the dust. It is configurable. See Configuration section for details.

3. Automatic Background Correction

MicroVigene™ automatically defines the spot single area from the background pixels. The regional background subtraction is an automatic and configurable process. See **Options::Spot**, In the **Configuration** section:



Background type: Variable and Flat background.



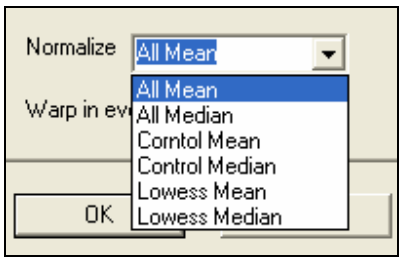
Configurable buffer zone width

Predefined the background percentile for background subtraction.

4. Normalization

MicroVigene™ automatically normalizes two images or two channels when showing correlation scatter plots and fold change lists. It is a normalization at spot intensity level.

MicroVigene™ supports linear and non-linear normalization, global or control gene normalization. Choosing a type of normalization from the **Normalize** dropdown list at **Options::Output** tab:



Global linear Normalization:
Select **All mean** or **All median** to normalized two image or a two-channels of composed image with their global mean or median intensity value.

Positive Control linear Normalization
Select **Control Mean** or **Control Median** to use only the mean or median intensity values of control genes (or positive-control spots) to normalize two images.

Note: The positive-control genes are specified in the platemap file under control column, see ID Name and Platemap Editor section.

Non-linear Lowest Normalization:
Select **Lowest mean** or **Lowest median** to use non-linear normalization. It can be used to correct un-even response due to the level of spots intensity. The normalization will use the results of the curve fitting of the correlation of two images.

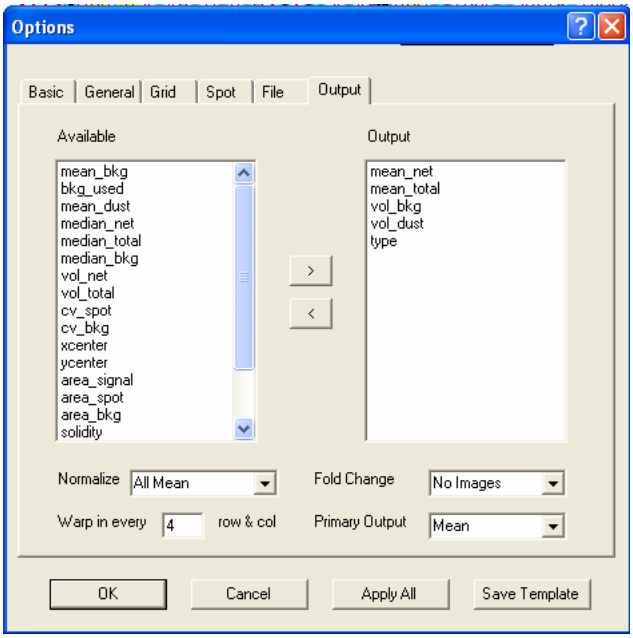
Note: The reference file in the normalization need to be pre-defined at the **Reference Has** token text fields at the **Options::File** tab. See Configuration and Set up section.

V OUTPUT DATA FILE

1. Selecting Output Data Set

There are 24 types of quantification and qualification pieces of data for a single image spot as shown in the following screen capture:

Select the menu dropdown list, **Application Menu::Analysis|Options**, or click on the Toolbar:



At **Options::Output** tab: use > and < button in the middle to select **Output** data. Each variable will be one column in the output file in the order selected in this output box.

- area_bkg---background area, which excludes dust area
- area_signal--- pixels in signal area are used to calculate the intensities
- area_spot---actual spot area found by program.
- aspect---aspect ratio of spot
- bkg_used---background value used to get net value, which is determined by percentile of spot background histogram.
- circularity---spot circularity
- cv_bkg---background coefficient of variance
- cv_spot---spot coefficient of variance
- dustiness---one of the dust index . It is the maximum pixel value that subtracts to spots mean value.
- mean_bkg---the average value of background signal

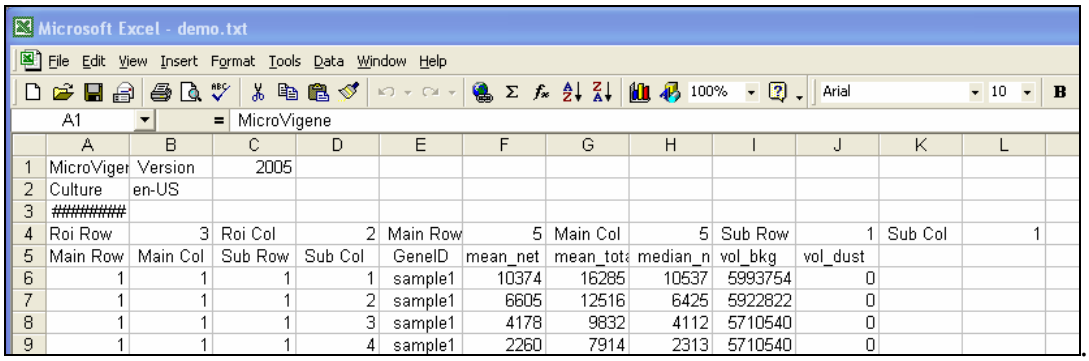
- mean_dust---the average value of dust
- mean_net---spot mean subtracted by background used and with dust removal
- mean_total---spots intensity mean value before background subtraction
- median_bkg---background median
- median_net---spot median subtracted by background used and with dust removal
- median_total---spot median
- solidity---solidity of spot
- type---spot type to mark the spot quality
- vol_bkg---background mean times background area
- vol_dust---dust mean times dust area
- vol_net---mean_net times signal area
- vol_total---mean_total times signal area
- xcenter---spot mass center x
- ycenter---spot mass center y

2. Saving Output Text File:

Click on the Toolbar or select **Application Menu::File|Save|Spot Info** to save the file.

3. Data Output .txt Format File

The output file is saved in the text format, which can be opened with Windows Notepad or Microsoft Excel.



	A	B	C	D	E	F	G	H	I	J	K	L
1	MicroViger	Version	2005									
2	Culture	en-US										
3	##########											
4	Roi Row	3	Roi Col	2	Main Row	5	Main Col	5	Sub Row	1	Sub Col	1
5	Main Row	Main Col	Sub Row	Sub Col	GeneID	mean_net	mean_tota	median_n	vol_bkg	vol_dust		
6	1	1	1	1	sample1	10374	16285	10537	5993754	0		
7	1	1	1	2	sample1	6605	12516	6425	5922822	0		
8	1	1	1	3	sample1	4178	9832	4112	5710540	0		
9	1	1	1	4	sample1	2260	7914	2313	5710540	0		

VI IMAGE FILE

1. Opening Image Files (.tif, .gel):

MicroVigene™ supports .tif , .gel and .img file formats. For good resolution, the 16-bit tif format is highly recommended. MicroVigene™ works better with 16 bit than with 8-bit format images.

1) Select the menu dropdown list, **Main Menu** or **Application Menu::File|Open|Image,** or click on the Toolbar to open an image file.

2) Select any folder and any image file in .tif, .gel and .img files from the browser. See Quick Start section for detailed information.

Image folder can be in any folder in the local or network drive. Since in the default, the .vgs files and .txt files will be saved in the same folder as the image files, permission is needed for reading and writing for that folder.

Both ways of loading image files, from menu list or button, will open a new sub-window for each image file.

Same images can be opened many times and each time you will open a new sub-window
Multiple image files can be loaded at the same time, by holding down **Shift** or **Ctrl** key when clicking on the files' names. Each image file will have its own window opened

2. Saving Image

MicroVigene™ saves images in standard 16-bit tif format by default.

Save Whole Image:
Select the menu dropdown list, **Application Menu::File|Save|Image,** to save the image under the same image name. It overwrites the original tiff imagefile.

Save ROIs Image:
Select the menu dropdown list, **Application Menu::File|Save|Roi Images** will save each ROI in an individual image file with a file name appended a roi order number, like: *-_1.tif.

Save Image in Other Format:

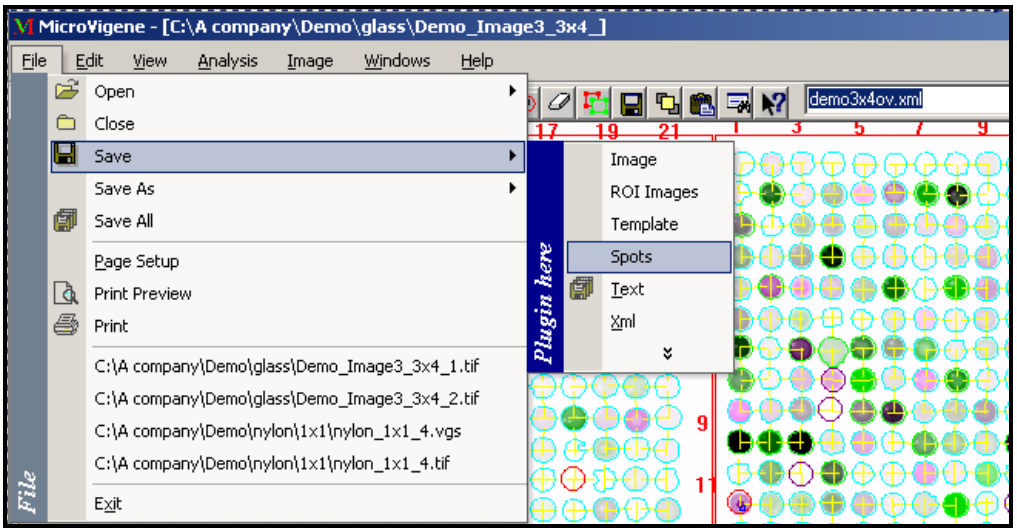
Export an image in other format can be done by fitting the whole image in a single ROI, then using **export** function for this ROI to save the image in other formats such as jpeg, png, gif, and bmp. See ROI Configuration session.

VII GRID/SPOTS FILE

MicroVigene™ will place Roi frames, grid lines, and spot boundaries on top of the image. These frames, lines, and spot boundaries can be saved as a **.vgs** MicroVigene™ Spot file. It can be retrieved later along with the image**.** The configuration setting information is also saved in the .vgs file.

1. Saving .vgs File

Select the menu dropdown list, **Application Menu::File|Save|Spots,** to save grid/spots file in the same name, as its image file, with .vgs extension.



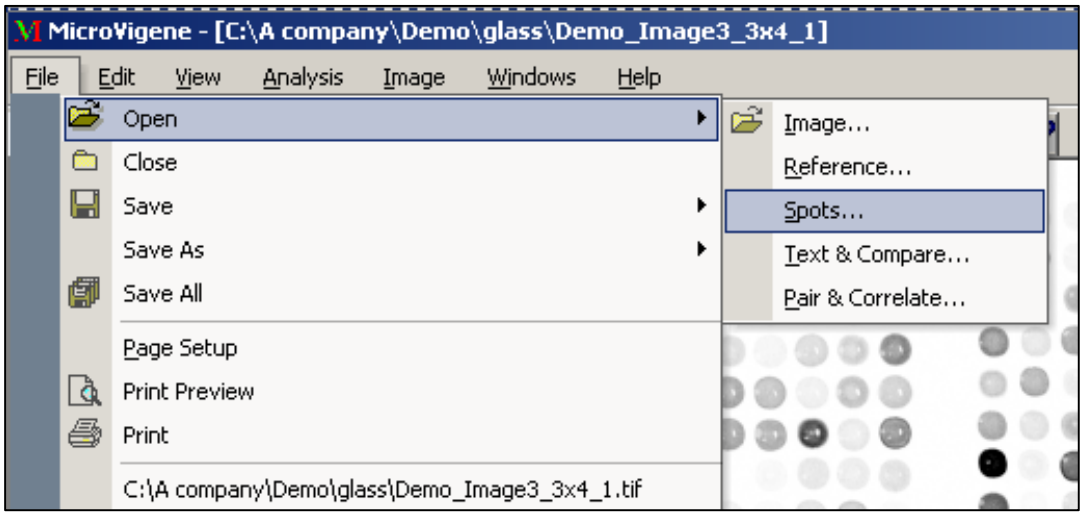
Click the button on the Toolbar; it will save both a spot file and a text file.

Important: When an image needs to be rotated and/or flipped before analysis. This new orientation of the image needs to be saved since the vgs file only associates with the new image but not with the original one. See Image File section for detail information about save image file.

2. Opening .vgs Files

Loading vgs in Opened Image Window

Select the menu dropdown list, **Application Menu::File|Open|Spots,** to load the **.vgs** file. Place the Roi, grid, and spots on top of the image.



Note: The **.vgs** file also contains most of the configuration setting information. If the template name is the same as saved in .vgs file, after loading the spot file, the configuration setting for this window will be updated by the .vgs file. If you want to use the setting in a template file, after loading the vgs file, reselect the template file in the template drop down box.

Directly loading a vgs File

Click on the Toolbar. Select .vgs in the file type field in folder browser. The image file with the same name, but extension in a .tif or .gel file, will be loaded first. Then the **.vgs** file will be loaded. It will open new sub window for this image and vgs files.

Note: If there is no same name image file in the same directory or folder, vgs files will not be loaded.

Open Multiple vgs Files

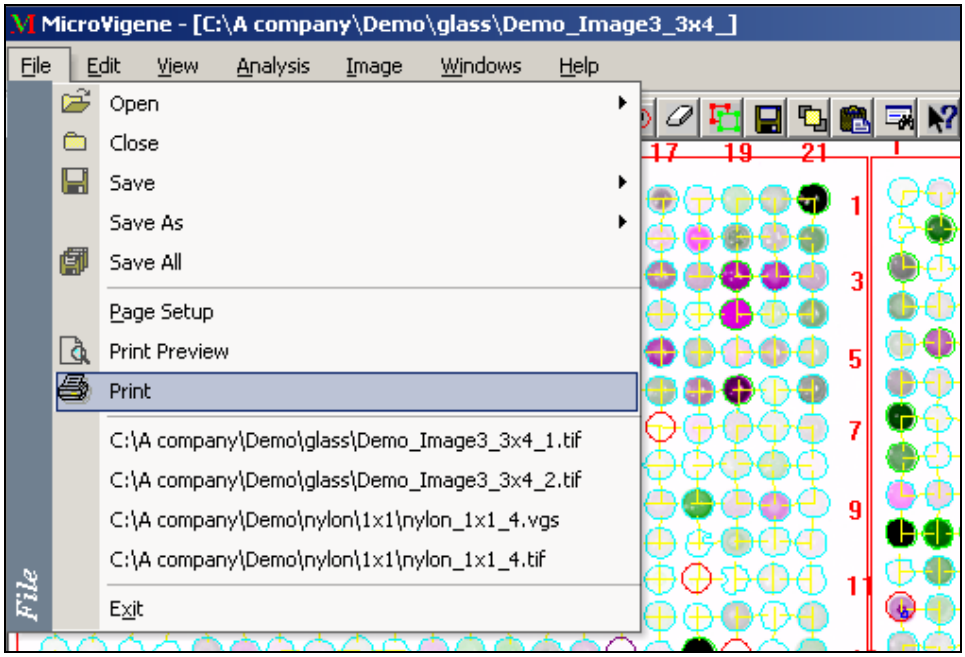
Select multiple vgs files by holding down the **Ctrl** key or the **Shift** key. A sub window will open for each vgs file.

3. Printing Image and Grid/Spots

MicroVigene™ provides print feature for image with spot **.vgs** files. It is particularly helpful for batch process. If there are any missing alignments or spots, it can be easily identified from the print out.

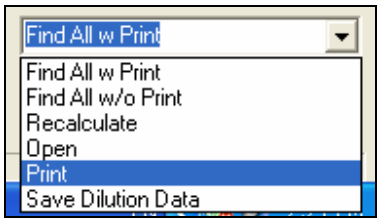
Manually Print:

Select the menu dropdown list, **Application Menu::File|Print** or clicking the button on the Toolbar to print out an image with the ROI frames, grid lines, and spot boundaries in the selected sub window.



Batch Process Print

It is supported in batch processes: **Main Menu** or **Application Menu:Analysis|Batch….** Select files from the **Add Files** button to open the file browser. Then select **Print** from the operation drop down list. Click the **Run** button to automatically print out a list of vgs files.



Note: Before launching MicroVigene™, always check printer connection. The simplest way is to use any other application (such as a Microsoft Word program) to print out a test page.

VIII VIEW OUTPUT DATA

MicroVigene™ has built-in features to allow you to visualize the image analysis process. The output data for any spot can be associated back to the Comparison table with text data. It allows you to verify any spot intensity to spot image in 2D and 3D format, so you can make sure the values for the spots are only from the spot area pixels. Any little changes between spots can be visualized in 3D view window.

The current version has the following features:

Viewing list of spot info

Viewing text and comparison

Viewing correlation

Viewing fold changes

1. Viewing Spot Information

Select **Application Menu::View|Spot Info**. See Review Data Quality section.

2. Validating Reproducibility, and Comparing Data from Other Application

For a same image, use a scatter plot to compare data quality with different methods. The data comparison is in spot mean or median intensity value.

Step 1: Choose intensity type for the scatter plot:

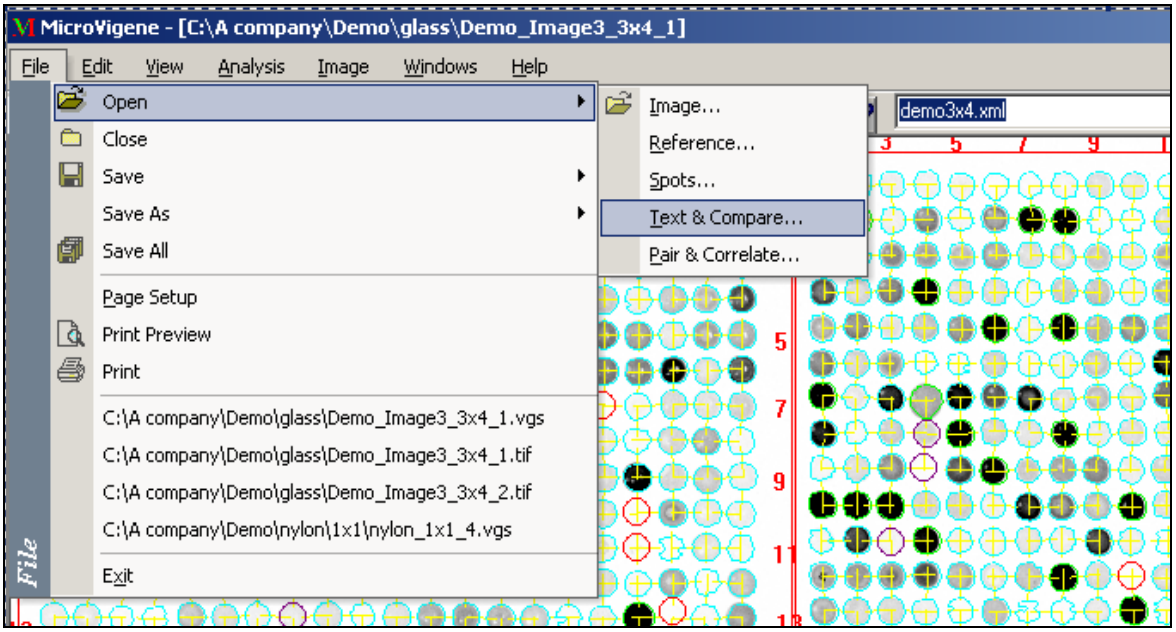
Select either **mean** or **median** from the dropdown list of **Options::Output|Primary Output**. See Output in Configuration Section.

Step 2: Load and analyze the image. See Quick Start section.

Step 3: Text & Compare:

Select the menu dropdown list , Application Menu:File|Open|Text & Compare. Select a text file from any folder to compare.

Note: The text file format should be the same as MicroVigene™ text output format. Other format data sets can be supported via plug-in.

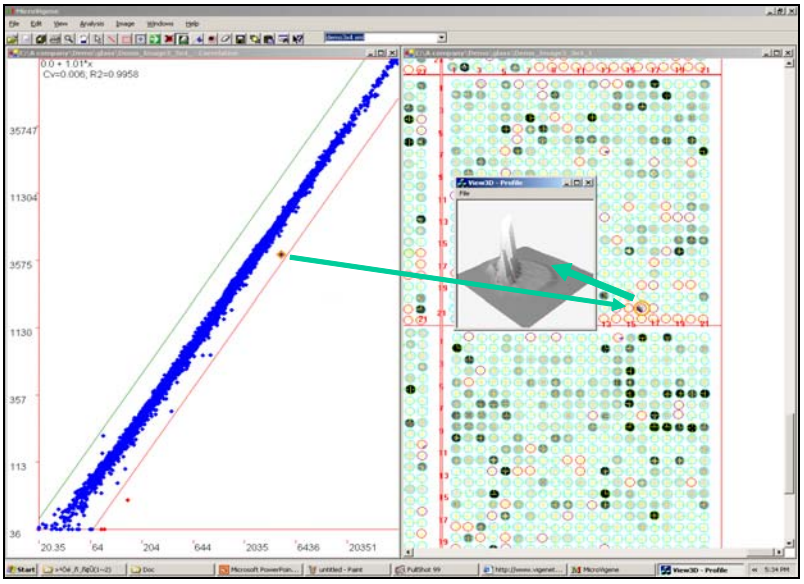


Step 3: Viewing the Text and Comparison

MicroVigene™ Text & Compare feature is loading another set of data to compare with the current data in the memory, which will be analyzed by MicroVigene™. It provides a correlation scatter plot for these two sets of data from the same image. For each spot, it will have a dot on the scatter plot with its **X** value from the loaded data set and **Y** value from the memory. The dots on the scatter plot and the spots in the image are associated with each other. Clicking a dot on the scatter plot will highlight the associated spots on the images, as shown in following screen capture.

Note: The association is active when the window is selected. Use on the Toolbar to select the window.

The scatter plot is in log scale. There are three lines in the scatter plot. The middle **blue line** is the best linear fit for all the spots. The **Green line** is the 2 fold up regulate and the **red line** is 2 fold down regulate. **Blue dots** represent spots that have less than a 2 folds intensity difference, and **green dots** represent a 2-fold up regulate and **red dots** represent a 2-fold down regulate. MicroVigene™ data is always used as sample data and other loaded text data is used as reference for comparison.



Comparing data in text format can offer:

Data Comparison with itself to Verify 100% Reproducibility.
Simply load previous saved MicroVigene™ output text files for the same image.

Comparing Different Output Data From Various Venders
Load text data from other venders. The text layout may be changed to suit this feature. It needs to be the same format as the MicroVigene™ output text format or a supported format.

Comparing Data Accuracy with Data From Statistic Analysis
Load text data from statistic analysis results. The text layout needs to be checked first to perform this comparison.

3. Correlation Between Two Images

View a scatter plot of correlation between two individual images or two channels.

Step 1: Set up Options Dialog Box
See the instruction in Output or File in Configuration sections, to set up.

Choose intensity type:
Select **mean** or **median** from dropdown list of **Options::Output|Primary Output**.

Define the Reference File
Enter reference name token at **Options::File|Reference has.**

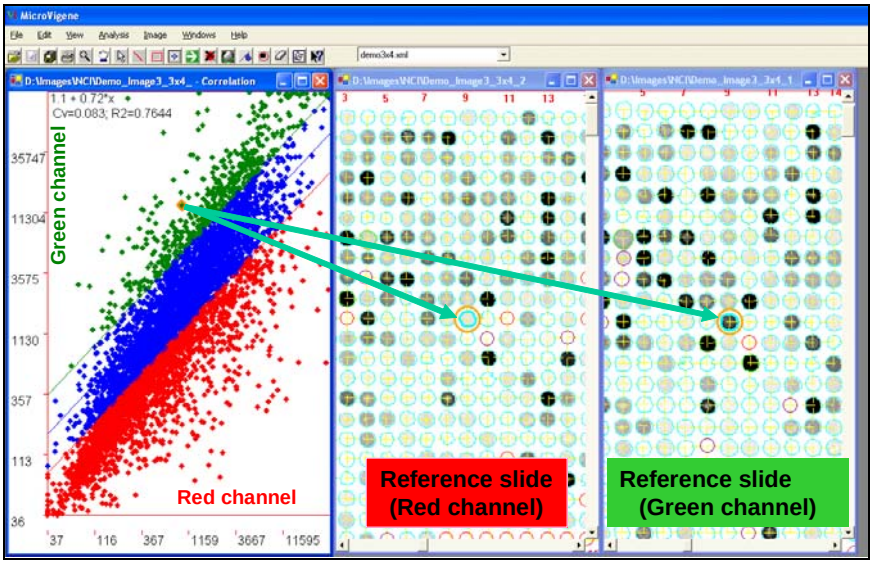
Normalization:
The data in correlation view is normalized. Choose a normalization type from the dropdown list of **Options::Output tab.** .

Step 2: Load two image files into two individual child windows in MicroVigene™, then analyze them individually, or load two **.vgs** files of these images. Also could load a composed two channel image into one window then do image analysis.

Step 3: Select the menu dropdown list, **Application Menu::File|View|Correlation.**

Step 4: View the correlation of these two images.

MicroVigene™ provides associated correlation scatter plot for two images. When selecting to view a correlation feature, it will display three vertical tiled windows(in the order from left to right: scatter plot, reference image, sample image). Each dot in the scatter plot is associated with a spot in the image window. Each window can be operated independently. Click on the top of the window to highlight the window in order to be activated.



Scatter Plot

The scatter plot is in log scale. There are three lines in the scatter plot. The middle **blue line** is the best linear fit for all the spots. The **Green line** is the 2 fold up regulate and the **red line** is 2 fold down regulate. The **Blue dots** are the spots different in intensity that are less than 2 fold, while the **green dots** are 2 fold up regulate and the **red dots** are 2 fold down regulate.

4. Folder Changes Between Two Paired Images

Viewing a Fold Change List between Two Channels:

Step 1: Set up Options Dialog box

The steps to choose intensity type, define reference file and select normalization type are the same as "Correlation Between Two Images" in this chapter.

Select Output Spot Image
Select the spot image option, **No Images**, **Composite Only** or **All images** from the **Options::File|Fold Change.** See Output in Configuration section.

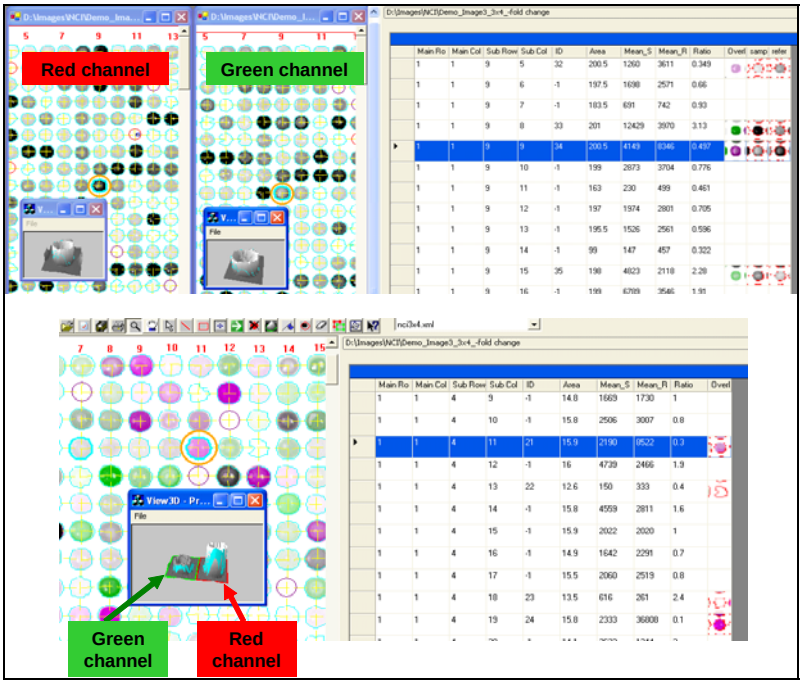
Step 2: Load two image files or one composed image and analyzed as the same step as "Correlation Between Two Images" in this chapter.

Step 3: Select the menu dropdown list, **Application Menu:File|View|Fold Change.**

Step 4: Viewing Folder Change List

When selecting a view fold change feature, MicroVigene™ will display three vertical tiled windows (in the order of left to right-reference image, sample image, Fold Change list). Each row in the fold change list is associated with the spots on the images in the left two windows. Each window can be operated individually. Click at the top of the window to highlight the window in order to activate the window. Click on the far left gray column in the fold change list for each row; the row will be selected and highlighted as well as the associated spots on the images.

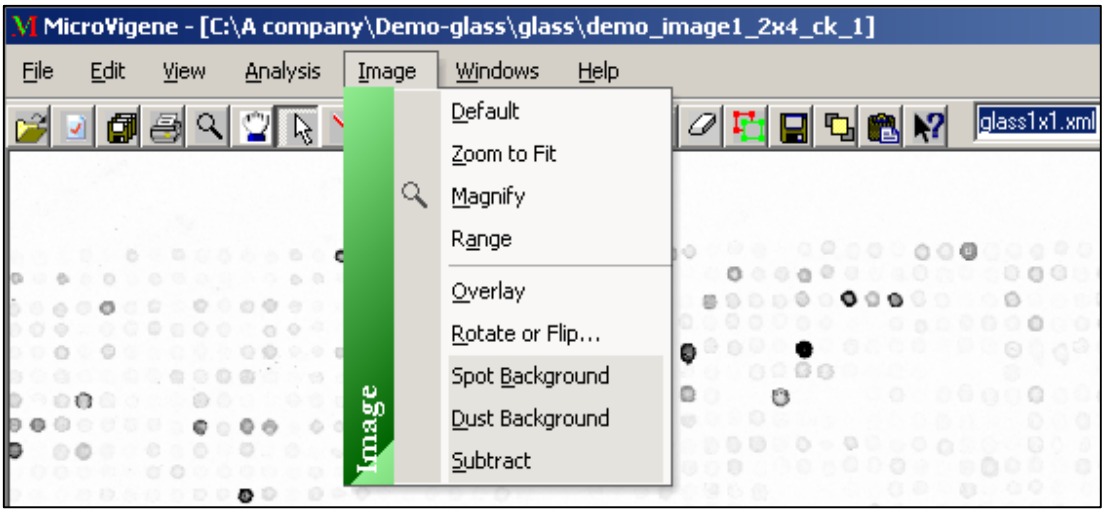
View Fold Change between Two Single Channels Images and Composed Image:



Data listed in Folder Change List are the values of detected signal area, intensities of the samples and references, their intensity ratio, and the option of having a spot image in a single channel or an overlapped image.

IX WORKING WITH IMAGES

There are sets of tools that are available for working with rows and analyzed images:



See the "Review Data Quality" Section for following function :
Application Menu::Image|Default:
---Display the image in original pixel resolution**.**

Application Menu::Image|Zoom to Fit
---Zoom the whole image to fit to the screen window.

Application Menu::Image|Magnifier
---2D magnify feature.

Application Menu::Image|Range

-- Open the Image histogram window to adjust image contrast and invert the image.

1. Image Rotation and Flipping

In MicroVigene™, you can rotate and flip the image and different orientation and directions.

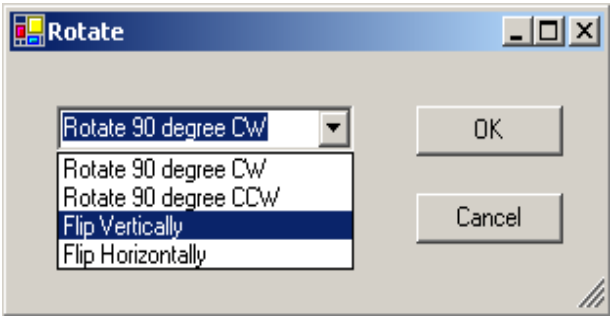
Select the menu dropdown list, **Application Menu::Image|Rotate**, to pop–up **Rotate** dialog box. Select one option in the **Rotate** dialog box:

Rotate 90 degree CW (clock-wise)

Rotate 90 degree CCW (count-clock-wise)

Flip Vertically

Flip Horizontally



There is no time limitation for rotating and flipping.

Important: Analyze an image after rotating and flipping. The spot file is only associated with the image after rotating/flipping. If at a later time, retrieving the saved grid/spots file .vgs is desired, it must have the associated image opened first. It is recommended that the image is to be saved after rotating/flipping.

2. Images Overlay

MicroVigene™ supports direct image overlay for **glass** platform images, and warping, as well as registration image overlay for **nylon** platform images.

Direct image overlay

For multi channel images such as glass slides, cy3 and cy5 images, there is relatively limited shift in spot locations between 2 images. Since it is from the same slide and just scanned differently, such images can be overlaid directly.

Warping and registration image overlay

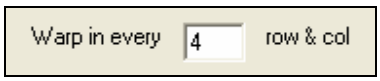
If two images are obtained from two different slides, most likely, the spot locations are shifted. Such images (like those of the nylon films) cannot be directly overlaid. The warping and registration manipulations must be used.

MicroVigene™ will automatically warp/register images based on found spots. The number of spots used as control points are determined by the number in the **Warp** field. We recommend setting every 4 rows and columns. Small number yields better registration but may take a long time. MicroVigene™ will use only strong spots as control points to register images if the number is set at **0**.

Step 1: Set up in Options Dialog Box

Reference file definition steps are the same as "Correlation Between Two Images" in **View Output Data** section.

Select registration and wrapping base unit.
Select the number of rows & columns as registration and wrapping overlay base unit.



For images that are not aligned, such as one in the nylon platform, grid/spots can be found first for each individual image, and then overlaid. MicroVigene™ will automatically warp/register images based on found spots. The number of spots used as control points are determined by the number in the **Warp** field. We recommend setting every 4 rows and columns. Small number yields better registration but may take a long time. MicroVigene™ will use only strong spots as control points to register images if the number is set at **0**.

Step 2: Load and analyze two images as the same step in "Correlation Between Two Images" in **View Output Data** section.

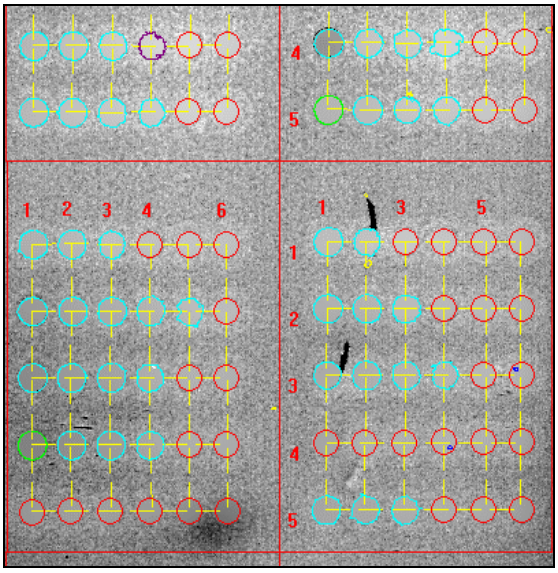
Step 3: Select the menu dropdown list, **Application Menu::Image|Overlay**.

3. Spot Background Image

Based on the spot segmentation, spot pixels are removed inside the boundary of each spot. This manipulation can be performed by selecting the menu dropdown list, **Application Menu::Image|Spot Background.**

Note: This function can only be performed when the image is analyzed.

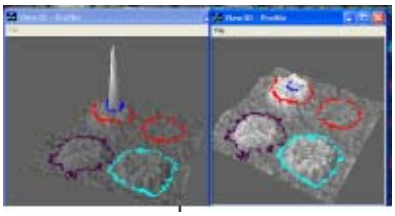
The following image is an example of an "Image without spot pixels":



4. Dust Background Image

Based on the dust detection, detected dust is removed with backfill. From the drop-down menu: **Application Menu::Image|Dust Background**, it will open a new window with an image that has the dust removed.

Note: This function can only be performed when the image is analyzed.

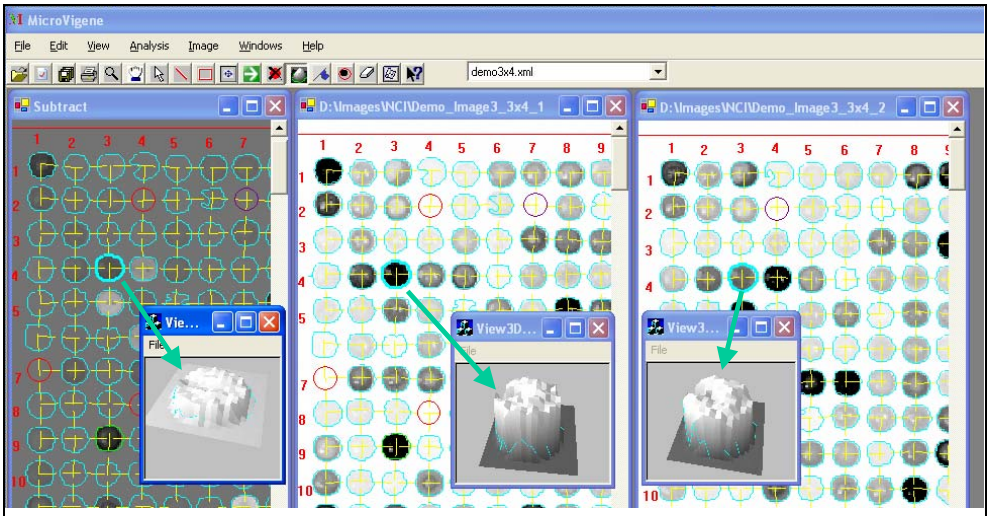


5. Two Images Subtraction

MicroVigene™ creates a new image in a new window by subtracting two images in their pixel gray levels.

Note: This function can only be performed when the image is analyzed.

Select **Application Menu::Image|Subtract.** As the example shown below, when Image I is currently active or highlighted window, then the subtraction function is launched from Image 1 window, so that the subtracted image is Image 1 subtract Image 2.



Subtract image Image 1 Image 2

X Image or Slide Barcode

MicroVigene™ can read the barcode on the image or on the slide. It can be read in while loading the image file and also can use the menu list, View|barcode to read the barcode in the barcode ROI.

Define the Barcode ROI

After loading the image, click barode icon on the toolbar. Move the cursor to the image, click and drag cross the barcode area on the image. Make sure the barcode roi covers the both ends and also includes some background.

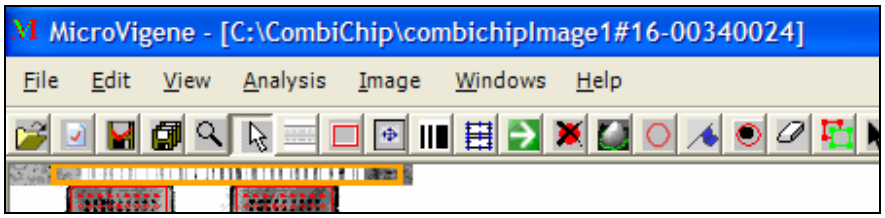
Define the Barcode format and directions

In the **Option::General** tab, define the barcode format, color of the background and direction for the barcode.

Note: The barcode ROI and definition of format, direction and background will be saved in a template.

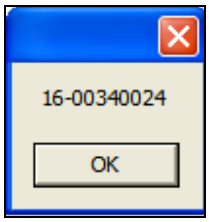
Auto Read in Barcode

When the template, which has the barcode ROI defined, is pre-loaded, the barcode will be read in while loading the image file. The barcode will be shown up at the end of image name at the top of the screen, started with #.



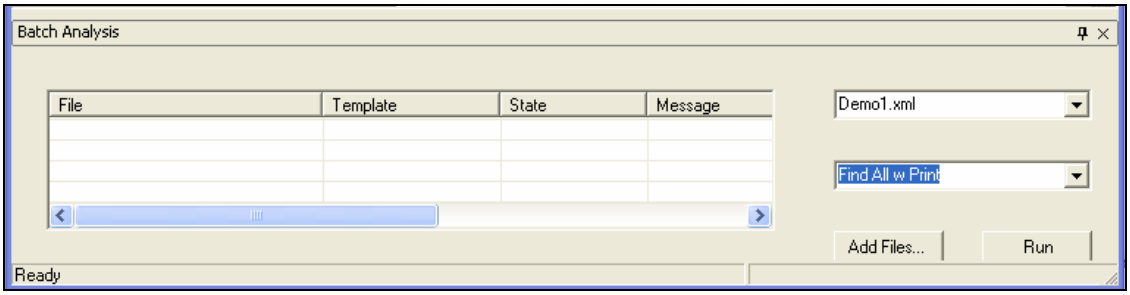
Display Barcode.

Select manu list , **View|barcode**, from Application window, there will pop up a barcode dialog box.



XI BATCH PROCESS

MicroVigene™ supports unattended batch analysis. Select **Application Menu::Analysis|Batch…** to open the **Batch Analysis** window at a low screen.



1. Setting Up

Adding Files to List

1. Click the **Add Files** button to bring the file/folder browser for selecting image files to the batch process.

2. Select a file(s) from any folder, either a local driver or a network folder (***Reading*** and ***writing*** permissions are required for these folders).

Select Template From List

Each file in the same batch process can have the same or different template files. Although the image files can be in any folder on the network, template files should be in the same folder. The Template drop down box only lists the files in the folder currently defined in the configuration box, **Options::Output|Template** text field.

Same Template for all Images:

1. Select a template at template drop down list

2. Add files from the **Add Files** button.

Different Template for Different Images:

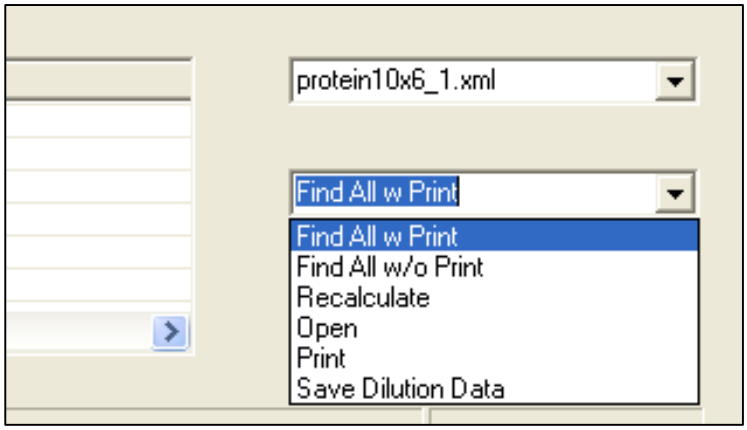
1. Add files from the **Add Files** button.

2. Select or highlight the file in the **File** list, then select a template in the template drop down list. The **Template** column in the file list window will be updated.

For group image files, hold down the **Ctrl** key, and then click file names in the **File** list to select a group of files. The template selected will be used for the entire group of image files.

Selecting Operation

MicroVigene™ provides several standard operations to automate the entire image analysis process. They are listed as below:



- **Find All w Print**: Find all and save the text and vgs files, and then print out the image with vgs.
- **Find All w/o Print**: Find and save the text and vgs files.
- **Recalculate**: Only re-quantify all the measurements.
- **Open**: Open image and vgs files.
- **Print:** Print out each image file and vgs files.
- **Save Dilution Data**: Find all and save dilution curve data.

2. **Run Batch Process:**

Click the **Run** button to run batch process.

Theoretically, MicroVigene™ has no limit on how many files can be run in the same batch process. During the batch process, MicroVigene™ opens one image file, analyzes and saves its **.vgs** and **.txt** files, prints the image and spot files (if the print option is chosen at set-up), closes the **.vgs** and image files, and loads the next image file to MicroVigene™. It supports as many images as desired in the same batch process.

Click to high light the image file names in the list for those only selected file to run the batch process. If no individual files are selected, it is equivalent to “select all”.

3. Status and Message

The **Status and Message Fields** will show the status of the batch process. Very often, when there is a warning message, a successful run is still achievable.

4. Stopping Batch Process

Click on the toolbar to stop the batch process.

XII DATA SECURITY

MicroVigene™ is in compliance with 21 CFR part 11 (Electronic Records; Electronic Signatures). MicroVigene™ supports user login security, signature and audit trail for data integrity protection. In MicroVigene™, however, it is optional to set up an audit trail.

1. **Setting up Signature and Auditor Trail:**

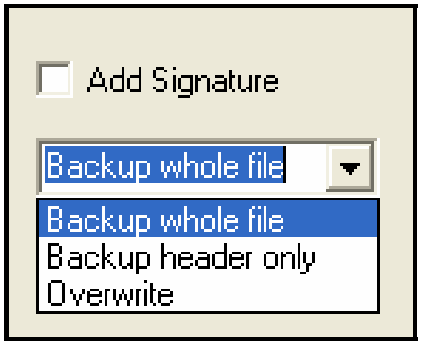
   Click button on the toolbar to launch Configuration, or the **Options** dialog box, and then click **Options::File** tab.

   Setting up Signature:

   Check the **Add Signature** check box. It will add user's signature into the file head in the output file. It also enables the Audit Trail process.

   Selecting Audit Trail Method:

   Select a file backup method on Audit Trail dropdown list::



Backup whole file:

Backup the whole text file with a time stamp at the end of the file name.

Backup header only:

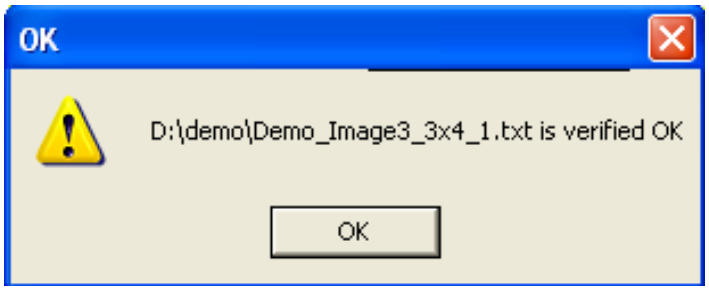
Back up the text file that contains only the file header with a time stamp at the end of the old file name.

Overwrite:

No backup up file will be created. The new content will overwrite the existing text file.

2. **Verifying Output File**
   MicroVigene™ version 2.0 has implemented a feature to verify if the output file is an original file .

   Select **Main Menu** or **Application Menu::Open|Text & Verify** to pop-up a file browser to verify a file. As a result, it will pop-up as shown below. If there is an error message, it means that the file has been modified by other application.



   Note: If the original saved file is in regular format and the current setting is “Audit Trail” enabled, **Text & Verify** will have an error message.

3. **Saving and Modifying the Output Text File**

   When the Audit Trail is enabled, the output file will be stamped at the top of each file. It cannot be changed or saved over by any other application, otherwise it will fail to the **Text & Verify** check.

	A	B	C	D	E	F	G	H	I	J	K	L	N
1	<!-- l0roQ8MsvxzvBr8yfJ5UMe7FinTuFQtW9txMk2zC9FO5mrgxX1LkTPcmlDCFY9weqXaPJ9mELzsSKRqwJalVog== -->												
2	<note time="2/8/2004 1:49:03 PM" user="UNREGISTERED" value="UNREGISTERED" />												
3	<note time="2/8/2004 1:49:22 PM" user="UNREGISTERED" value="Test for audit trail and signature" />												
4	MicroVigei	Version	1.275										
5	#########												
6	Roi Row	8	Roi Col	4	Main Row	2	Main Col	5	Sub Row	1	Sub Col	1	
7	Main Row	Main Col	Sub Row	Sub Col	GeneID	mean_net	mean_tot	vol_bkg	vol_dust	type			
8	1	1	1	1	sample1	1.33	34.3	29898	0	circle			
9	1	1	1	2	sample1	3.7	33.7	27180	0	circle			
10	1	1	1	3	sample1	2.84	31.8	26274	0	circle			
11	1	1	1	4	sample1	1.47	29.5	25368	0	circle			
12	1	1	1	5	sample1	1.81	30.8	26274	0	circle			
13	1	1	2	1	sample1	1.34	34.3	29898	0	circle			
14	1	1	2	2	sample1	3.58	34.6	28086	0	circle			
15	1	1	2	3	sample1	2.69	31.7	26274	0	circle			

Modify Existing Output Text:

It will verify the file first before saving new data over the existing file:

- If it is a MicroVigene™ saved file, i.e. it passes **Text & Verify** a pop-up message will show the reason for the modification entry. The file will be saved in the file header.
- Then it will save the existing file with the file name and a date /time stamp at the end. For example, protein1.txt will be saved as protein1~date-timestampe.txt.
- For multiple modifications at different time periods, it will list the entire file header to record all file history, but the data section will be replaced with newest data.
- If the **Text & Verify** fails, an error message will pop-up. You need to change mode to either **overwrite** in the **Options::File** dialog box or delete the text file, And then save again to generate the first version of text file.

XIII CONFIGURATION AND TEMPLATES

MicroVigene™ needs to be configured to analyze any array images. For the same configuration, it will output the same image analysis data. If the general pin geometry, array layout, and scanner resolution are the same, the configurations are very similar. Therefore, for an existing system, after finding the configuration for the first image, other images only need a very limited adjustment before analyzing. . Completed setting in **Options** can be saved in a template file in **.xml** file format.

There are tutorials in online help in MicroVigene™ to show how to set up templates for different images.

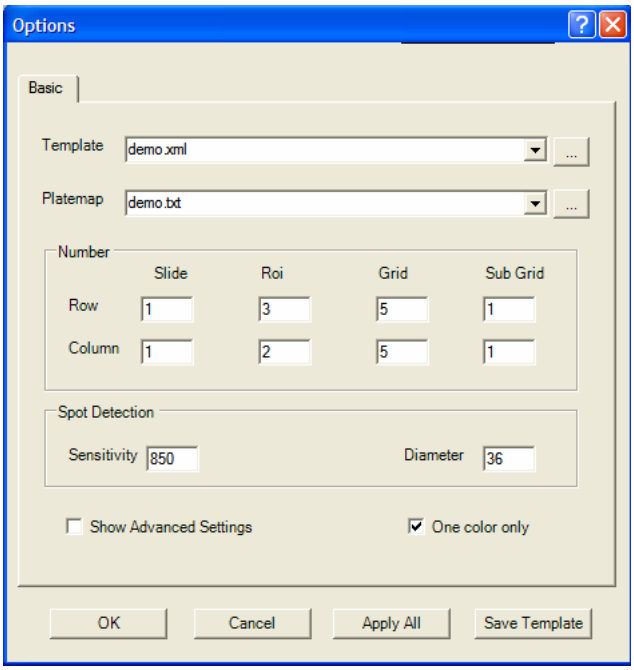
Launch configuration or Options dialog box:

Select **Main Menu** or **Application Menu::Analysis|Options**, or Click on the toolbar.

There are five tabs in the **Options** dialog box: **Basic**, **General**, **Grid**, **Spot**, **File and Output**.

1. Basic

Set up basic configuration under **Options::Basic** tab. For routine analysis, in most cases, you only need to adjust it for analyzing new arrays.



Creating new Template

Select a template file in the template folder or start from a default setting. Enter a new desired name in the **Template** text field. Make sure the type has ***.xml*** at the end.

Click **Save Template** button to create a new template.

Importing ID Name file: Platemap File

Select a platemap file from the platemap drop down list that associates each spot to a gene ID, a protein ID, or a sample ID. See **ID Name, Platemap Editor** for more information

Entering Array Dimension

In the **Number** section**,** enter array and grid dimension numbers.

- **Slide:** In the Row and Column fields, enter the number of rows and columns in the slides.
- **Roi:** In the Row and Column fields, enter the number of rows and columns in the ROIs in each slide.
- **Grid**: In the Row and Column fields, enter the number of rows and columns of spots in each ROI.
- **Sub Grid**: In the Row and Column fields, enter 1 for both fields. (Typical for nylon images with sub grid, 5x5, or 4x4).

Sensitivity for Weak or Strong Images

In the **Sensitivity** text field of the **Spot Detection** section, enter a number between 0-1000. For the majority of normal images, choose a number between 700-900.

Spots Diameter

In the Diameter text field of the **Spot Detection** section**,** enter the average spot diameter in integers of pixels or microns. A circle with this diameter is placed for any spot that cannot be found or for poor and weak spots. The setting in **Options::Spot** tab for maximum and minimum diameter will be automatically adjusted.

Spot Boundary Color

Check **One Color Only** check box to show all the spot boundaries in cyan color when image is analyzed. The good spots will be shown in actual spot boundary and other spots will be shown in circle as spot boundaries. Those circles will have the spots mass center as the circle center.

If **One Color Only** check box is un-checked, the spots boundaries will be shown in different colors according to the spot quality type. See Quality Determination Criteria in **Spot** section.

Advanced Setting Options

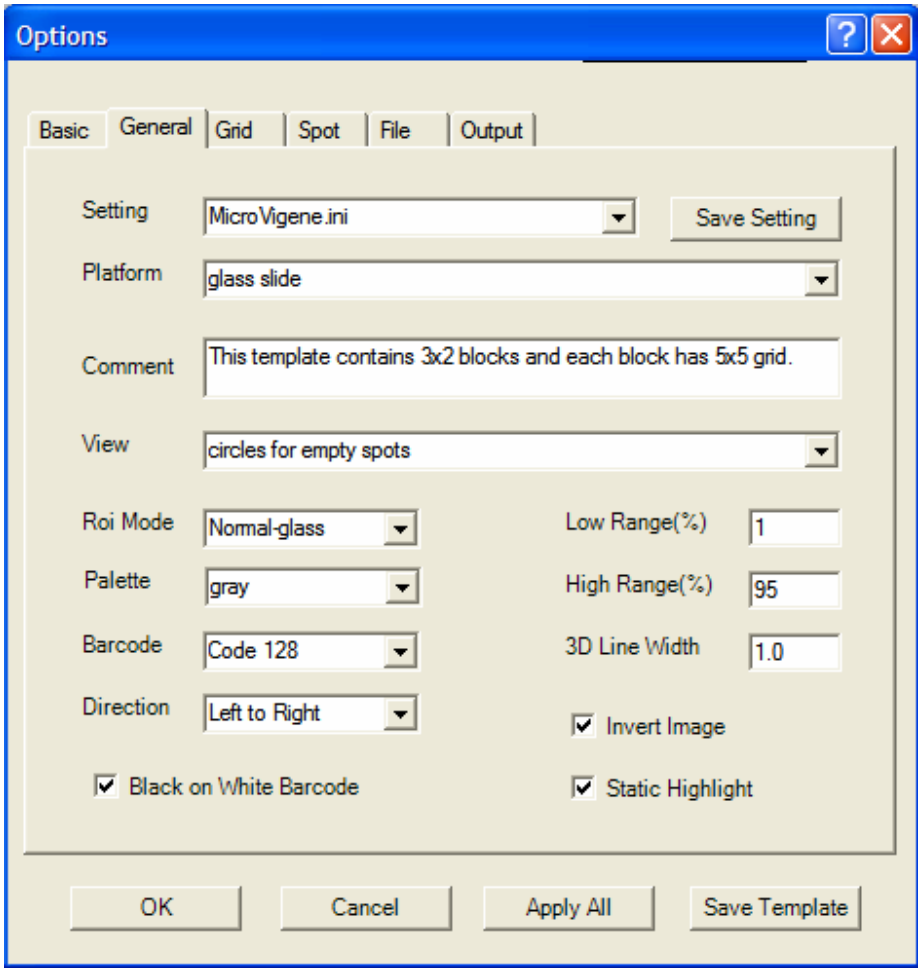
Click **Show Advanced Settings** check box to open another five tabs of advanced configuration options.

Saving Options to Template File or Activate Changes

After modifying any setting:

- Click **OK**: Modified settings only affect the current window. The changes will be lost when the image window is closes.
- Click **Apply All**: If modified settings are applied to all opened windows with same template files, the template files will not be updated.
- Click **Save Template** to save all the configuration settings to the template file. Without clicking this button, any changes will be lost after the window is closed.
- Click **Cancel** to cancel all setting changes.

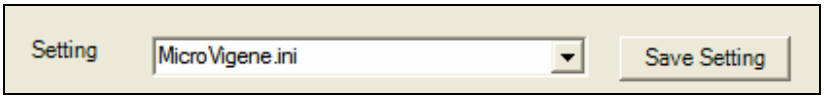
2. General:



Save Setting in Option box

The file path can be saved each time if there are different template and platemap folders used for different image sets. So switching between the .ini files allows you to quickly get back the file paths that your previous analysis used.

Click **Save Setting** before closing the **Options** dialog box. Give another name for different paths selection.



Glass or Nylon Platform

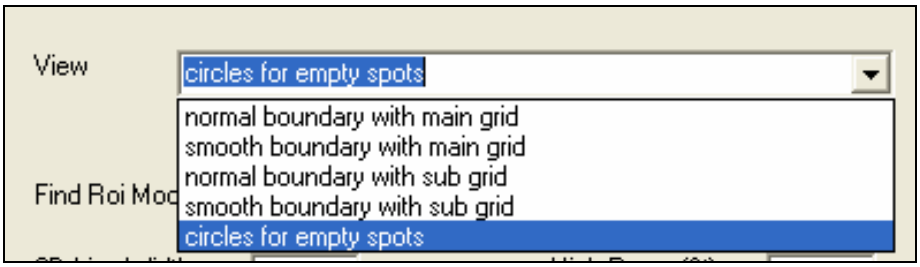
Select **glass slide** or **Nylon Film** at **Platform** drop down list.

Template Comments

In the **Comments** text box, record a comment.

Show Template Graphic Layout

Select **circles for empty spots** from the **View** drop down list to view the template layout before the image is analyzed. This is defined in the **Options** dialog box under **General**.


View
circles for empty spots
normal boundary with main grid
smooth boundary with main grid
normal boundary with sub grid
smooth boundary with sub grid
circles for empty spots
Find Roi Mod

Show Grid line and Spot Boundary

From the **View** drop down list, select one option of display of grid line and spot boundary when image is analyzed. For glass slide image, choose:

- Normal boundary with sub grid, or
- Smooth boundary with sub grid

Inverting Image or Image Contrast

Inverting Image:
Check this box to invert the display of images.

Setting up Image Contrast Gray Level Range

- **Low Range (%)**: Set up the image contrast to low range in percentage.
- **High Range (%)**: Set up the image contrast to high range in percentage.

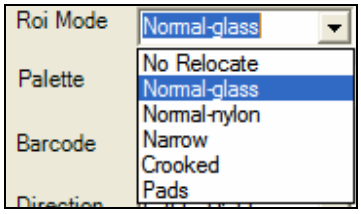
Note: These only change the display, and do not affect any pixel gray level in the image.

Spot Boundary Line in 3D View

Set up the line width of spot boundaries in 3D view display by entering a number of pixels in the **3D Line Width** text field. If it is set to –1 or any negative value, the spot boundary will use whole pixel as the spot boundary lines.

Automatically Define ROI Mode

In the **Find Roi Mode** drop down list, select one mode:



The following guidelines should be followed as below:

No relocate: For images with pre-defined ROI, do not use automatic process to find ROI.

Normal glass: For normal images with a wide and even ROI gap.

Normal nylon: For images with shifted grid and spots, large ROI and wide ROI gap.

Narrow: For normal images with a narrow ROI gap.

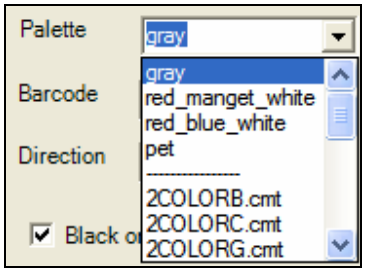
Crooked: For crooked and challenged images.

Pad: For 16- 96 pad or well images.

Note: For very strong and clean protein array, try to use the **Normal glass**, **Normal nylon**, **narrow** or **crooked** mode to automatically find the ROI. Or for an un-uniform array, manually define the ROI or adjust each ROI setting. Save the configuration with the ROI definition into the same template file and choose the **No relocate** mode to surpass automatically located ROIs processes.

Select Displayed Image Color

In the **Palette** drop down list, select a mode:

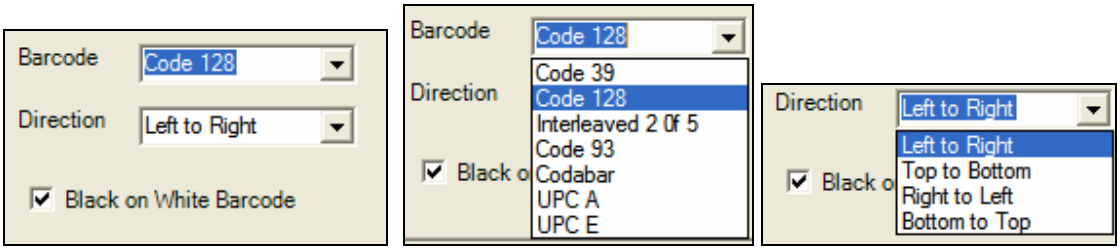


The image will change its gray color to selected color tune.

Note: The color schemas are predefined and should be loaded into the MicroVigene folder and in the lut sub folder. See **File** in the **Configuration** and **Template** section.

Automatic Read in Slide Barcode

MicroVigene supports reading in the barcode on the slide. The barcode has to be pre-defined in this section.



Select Barcode Background

At **Black on White Barcode** check box:

- Check: barcode is on the white background
- Un-Check: barcode is on the black background

Select Barcode Format

In the **Barcode** drop down list, select a barcode format:

- Code 39
- Code 128

- Inerleaved 2 of 5
- Code 93
- Codabar
- UPC A
- UPC E

Select Barcode Direction

In the **Direction** drop down list, define the barcode read in direction:

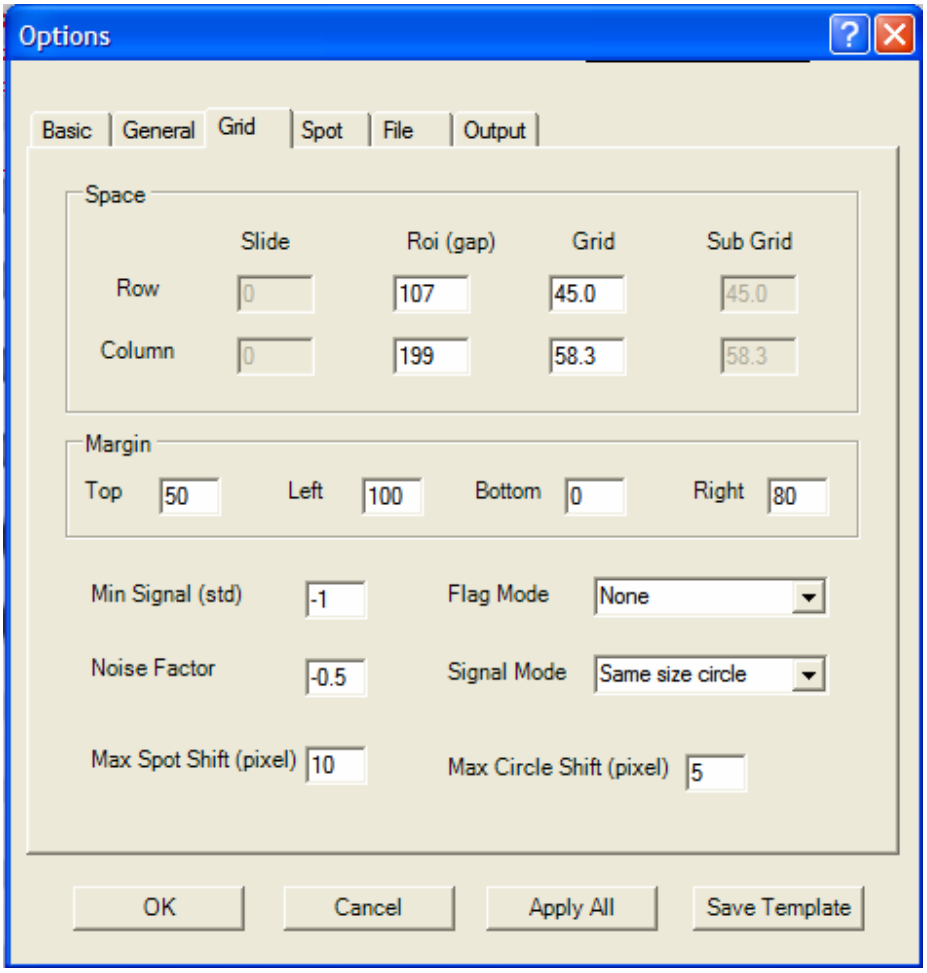
- Left to Right
- Top to Bottom
- Right to Left
- Bottom to Top

Spot Image Link format:

Static Highlight check box defines the selected or highlighted spot link from other window, such as Spot Info, correlation scatter plot.

- Check: Orange circle around the spot
- Un-Check: white-red dashed circle spins around the spot

3. Grid:



The parameters under the **Grid and Spot** tabs require the most adjustment for different array images.

Grid, Spot Spacing

In the **Space** section, enter all the spacing by measurements using on the toolbar. The units are all in pixels.

Slide: in the **Row** and **Column** fields, enter the vertical and horizontal slide (the data type is integer).

Roi: in the **Row** and **Column** fields, enter the vertical and horizontal ROI gap in (the data type is integer).

Grid: in the **Row** and **Column** fields, enter the vertical and horizontal grid spacing between the spots in a ROI.

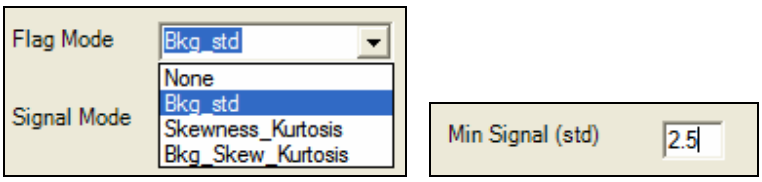
Sub Grid: in the **Row** and **Column** fields, the same value should be entered as the Grid Row and Column for the glass slide mode as Plateform, unless a sub-grid is used. In a nylon platform, typically there will be a 5x5 or 4x4 sub-grid.

Image Cut off Margin

In the **Margin** section, margins can be specified to exclude any undesirable area around the image border: **Top, Bottom**, **Left** and **Right.** For instance, the image may have some labels near the image border, which should be excluded from data analysis. The values entered are in pixels.

Auto Flag of Bad Spots

Base on the measurements of spots, set up threshold to exclude bad spots. The intensity and Skewness Kurtosis thresholds can be defined in **Flag Mode** drop down list with **Min Signal** (sd) text box.



Flag Mode in:

- **Bkg_std:** The value defined in **Mid Signal (std)** is the number of times of the spot regional background standard deviation. As the example listed above pictures, it means that any spot, whose intensity is lower than the 2.5 times of this spot background standard deviation, will be automatically flagged and its spot type will be changed to 6.

- **Skewness_Kurtosis:** Skewness is a measure of the asymmetry of a frequency distribution. A right, or positive, skewed forecast has a greater density of values occurring and the mode around the lower end of the range. A left, or negative, skewed forecast displays the opposite trend and can be developed using the beta distribution. Kurtosis is a measure of whether the data are peaked or flat relative to a normal distribution. That is, data sets with high kurtosis tend to have a distinct peak near the mean, decline rather rapidly, and have heavy tails. Data sets with low kurtosis tend to have a flat top near the mean rather than a sharp peak. A uniform distribution would be the extreme case. So it means a spot will be flagged as bad if its Skewness-Kurtosis exceeds the limits specified in the fields.

- **Bkg_Skewness_Kurtosis:** The value defined in **Mid Signal (std)** applies for both background standard deviation and spot Skewness.

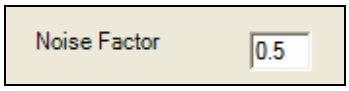
No Negative Output Intensities

If downstream analysis cannot take any zero or negative value of net spot intensities, set a small factor in **Min Signal (std)** field. So that, if the spot's **mean_net** or **median_net** is negative, the output intensity value will be replaced by its background standard deviation times this small factor. It is recommended to set this value equal or lower than **0.1**. It means that any negative net intensity spots will have an output value of one tenth of the background standard deviation. If this value is set at a negative number, MicroVigene™ will output the **true net intensity** values, which contain negative values. Default is –1.

Note: The **Flag Mode** needs to be set at none for this option.

Detect Very Weak Spot

Activate additional algorithm to increase the sensitivity to detect very weak spots by setting **Noise Factor** any value from 0 to 1. Any negative value will inactivate this fine analysis process.

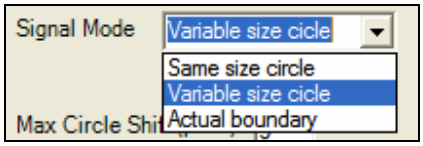


Actual, Variable Size Circle and Fixed Spot Signal Area

Select **Signal Mode** drop down list to define the spot pixel area used for spot intensity quantifications.

Select **Signal mode** in

- **Same size circle** to measure all the spot pixels the same size circle. The diameter is defined in the **Diameter** field in **Options::Basic**.
- **Variable size circle** to measure all the spot pixels the variable size circle. The spot size is the same as the actual spot, but is in circle shape and centered with spot mass center.
- **Actual Boundary** to measure the actual spot area



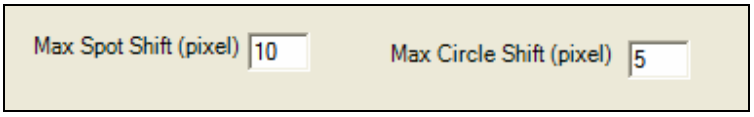
Limitation to Maximum Shift Grid

MicroVigene™ uses flexible grid according to the actual spots location.

Enter a limit of **Max Spot Shift (pixel)** to set the maximum spots' shift distance from the expected grid line. This setting applies all good (cyan), blooming (green) and poor (purple) spots.

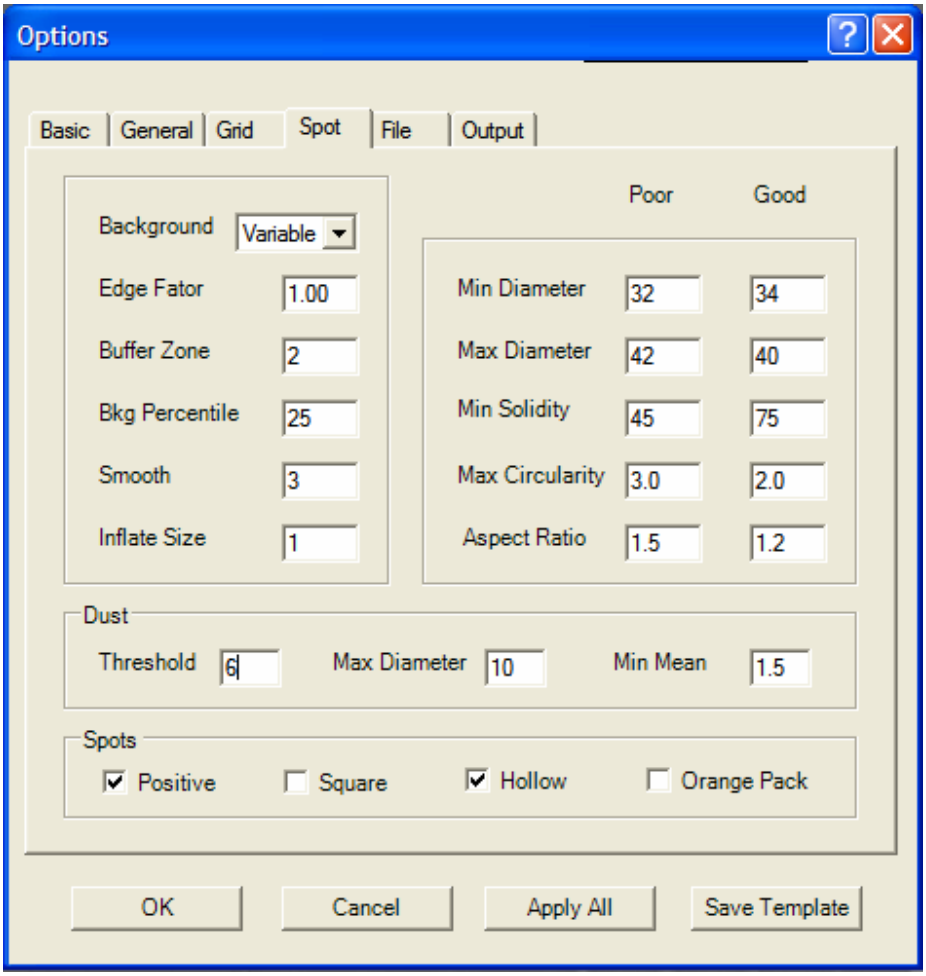
Enter a limit of **Max Circle Shift (pixel)** to set the maximum red circle spots' shift distance from the expected grid line. This setting only applies all red circle spots.

It should not be larger than half of the grid space. MicroVigene™ will make no shift from the grid line if it is set at "–1".



4. Spot:

All the settings in the **Spot** tab rarely need to be changed only the quality of image is quite different and needs more fine tunings.



Background Subtraction Mode and Buffer Zone

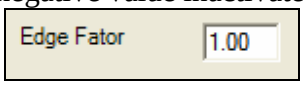
Flat or Variable Background:
Select **Variable** for true background images in most cases. For pre-cut or filtered image select flat.

Setting up Buffer Zone:
Enter an integer number, the number should be smaller than the [(grid space)-(spot diameter)]/2. Pixels in this zone are not accounted either for the spots or the background.

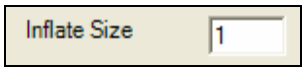
Selecting the Background Percentile Level:
For background subtraction, enter a percentage value of **Bkg Percentile**, in 0-100 to calculate the background value. A higher value means that a higher background value will be used.

Refine Spot Signal Segmentation

Edge Factor of Spot:
Enter a value from 0 to 2 to activate a refine analysis of spot segmentation based on the spot shape. As set at high value, the analysis looks for the best signal segmentation at high intensity pixels. For lower value, the segmentation will be close to the background. Any negative value inactivates this analysis.



Inflate Size of Spot:
Enter a number of pixel in **Inflate Size** text box to activate the analysis process to check if the segmentation could be lower close to background, or could inflate the spots boundary in such number of pixel larger. Any negative value inactivates this analysis.



Smooth Fuzzy Edge Spots:
In the **Smooth** text field: enter a number between 1 and 10. Increase the value to smooth out more noise. Default is 1.

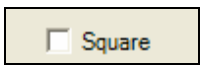
Note: This is only used in finding grid/spots. For spot measurements, you will still use original image data to calculate.

Defining Positive or Negative Spot Image

Check the **Positive Spot** check box when the spot pixel's gray level is higher than the background in the image, a positive spot image. See **Spot Info** section for how to define positive or negative spot image

Square and Circle Shaped Spots

M**icroVigene**^{TM} analyzes circle-shaped spot array image as well as Square-shaped spots. Checking **Square** check-box is to analyze square-shaped spot array. Unchecking this check box is for circle-shaped box.



Handle Hollow or Ring- Shape Spots

When checking the **Hollow Spots** check box, M**icroVigene**™ will treat non-solid, but it will also have ring shaped spots as regular spots do. If there are some ring-shaped spots in the image, check this box.

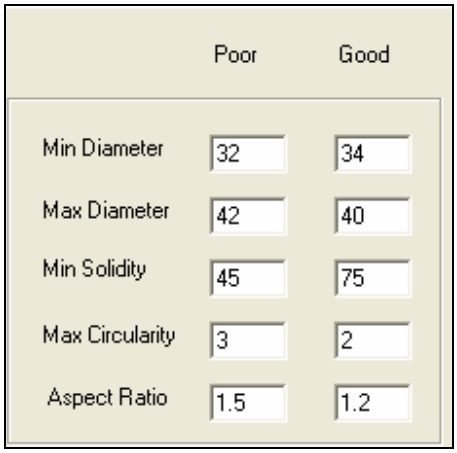
Orange-Pack and Regular Grid Array

When checking the **Orange Pack** check box, M**icroVigene**™ will grid each ROI in orange page array. Un-checking this check box is for circle-shaped box.

Fine Tune for Handling More Challenging Images

Following fields will be automatic updated with the setting of spot **Diameter** in **Options::Basic** tab. Fine tuning of these five category values is necessary needed to handle more challenging images.

Spot quality categorization for classification:



The Four spot quality classes are**:**

- **Blooming Spot,**
- **Good Spot,**
- **Poor Spot and**
- **Weak (circle) Spot**

There are five measuring criterions to categorize the spots. The good spots will meet all five criterions. The poor spots will have at least missed one limitation of five Good criterions. And weak spots have at least missed one of five Poor criterions.

The Five measuring criteria are:

Min Diameter Poor and **Good:**

Any spots with a diameter that is less than the **poor** value are filtered out and marked as a red circle are regarded as weak spots. Any spot's **diameter** that is in the range of being good or poor will be marked as a purple circle.

Max Diameter Poor and **Good:**

A spot is considered as a blooming spot if the diameter is larger than the **good** value and its intensity exceeds a certain value or the **diameter** is larger than the **poor** value.

Min Solidity: Poor and **Good:**

It is the percentage ratio between the spot area and the minimum convex area. Imagine putting a rubber band around the spot, the area inside the rubber band would be the minimum convex area.

Max Circularity: Poor and **Good:**

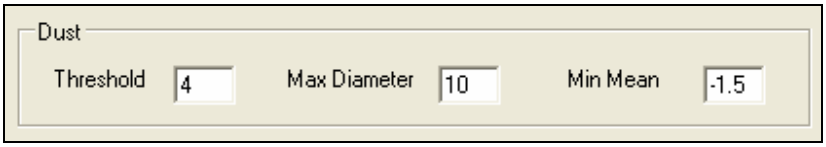
Define as (Perimeter^2)/(4pArea), which is 1 for circle and > 1 for any other shape. Circularity measures the shape roundness plus the boundary roughness.

Aspect Ratio: Poor and **Good:**

Define the aspect ratio for poor and good spots. 1 is for circle and > 1 for any other shape.

Dust Removal and Contamination Flag Options

The automatic dust find/removal and Contamination Flagging can be configured to exclude only dust but not single pixels. By setting all three parameters as explained below, MicroVigene™ will only detect and remove the type of the dust you want to remove.



Threshold:

Define the threshold for dust detection. The value is from 0 to 100. 0 or –1 will turn off the dust detection function. The normal setting is between 4 and 10. "**–1**" will turn off the dust removal function.

Max Diameter:
Define the maximum dust size in diameter. If the dust found has a diameter that exceeds this value, it is not considered as a dust anymore.

Min Mean:
Define the sharpness of the dust. Small value is for detecting small size peaks. In most cases, 0.5 can detect very small dust peaks.

Auto flag contaminations:
Set **Min Mean** to negative value, like –1.

5. File

All files can be anywhere, either in the local drive or on a network drive, as long as MicroVigene™ has full *read* and *write* access permission. Click the **browser …** Button at end of the file folder entry text box to brows the folder.

Selecting Files Folder or Path

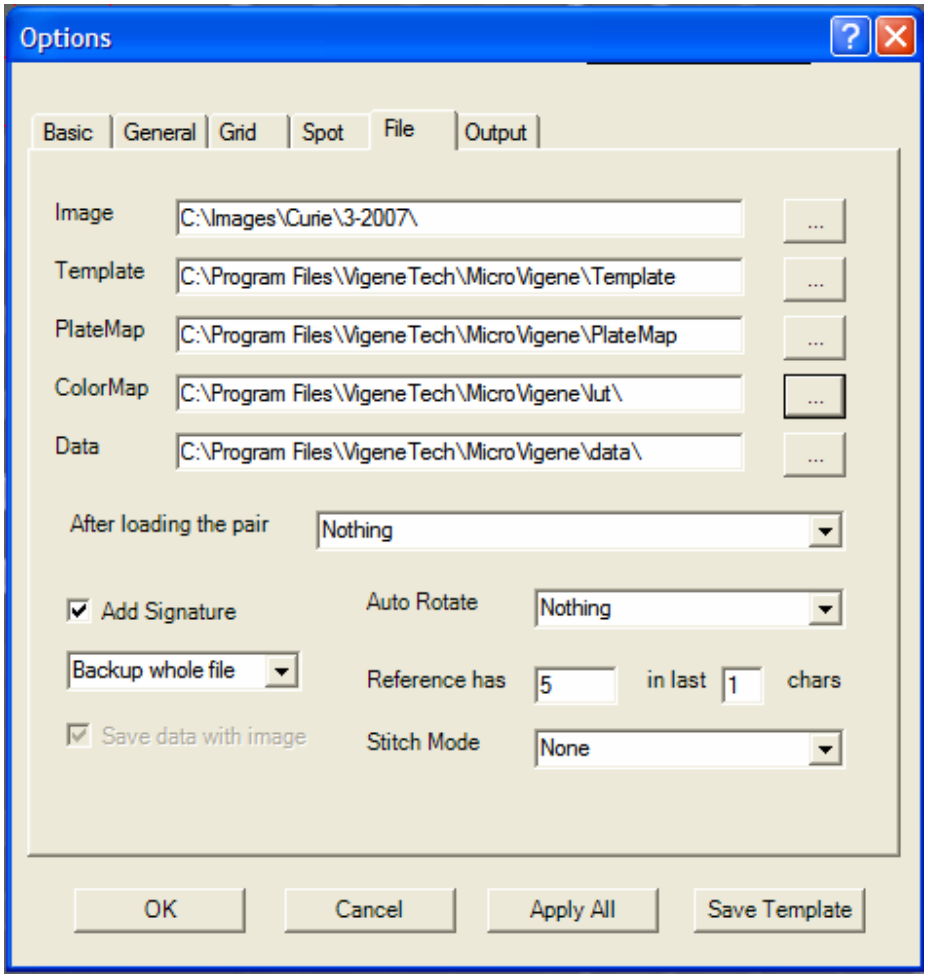


Image:
Select an image file folder from the browser.

Template:
Select a template file folder from the browser. Only .xml files in that folder will be shown in the **Template** drop down list.

Plate Map:
Select a platemap file folder from the browser. Only .txt files in that folder will be shown in the **Platemap** drop down list in **Options::Basic.**

Color Map:
Select a color map file folder from the browser. Only .cmt color files in that folder will be shown in the **Palette** drop down list in **Options::General.**

Data:

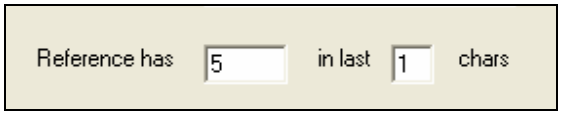
In the default, all image analysis results files will be saved at the same folder as the original image is. Saving the results file in another folder, it can be defined by using the file browser select.

Note: This option currently is not active yet.

Define Reference File for Data Analysis

The **Reference** has: a text field to specify the reference token and where (last number of characters) to look for the token. For above screen capture, for an image file with "5" in last "1" characters, MicroVigene™ will automatically assign it as a references file.

Before loading an image, the reference file name pre-fix needs to be defined in the configuration dialog box, **Options::File|Reference** has as following

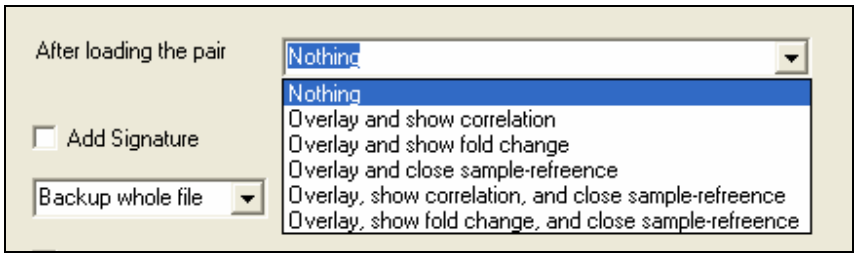


It means, any file name with 5 at last 1 character will be the reference file. Like any file in format of XXXXX….XX**5**.tif will be the reference (**X** value of the dot in scatter plot) and other will be sample file (**Y** value of the dot in the scatter plot).

Note: If two images need to do their correlation, fold change and image wrapping overlay, the reference file needs to be defined here before the image files are loaded. See Data Analysis section.

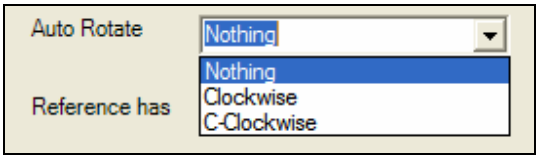
Auto Loading Data After Load Image .vgs Files

MicroVigene™ will automatically load other data or overlay the images after loading the image and .vgs files. Select a post load operation from **"After loading the pair"** drop down list for after loading the sample-reference pair.



Automatic Image Rotation

Select CC or ACC from the **Auto Rotate** drop-down list if an automatic rotation is wanted after loading an image. MicroVigene™ will check image orientation and if it is different from the template file, MicroVigene™ will rotate the image as directed by setting determined here. See more information about rotation in the **Working with Images** section.



Activate Signature and Audit Trail

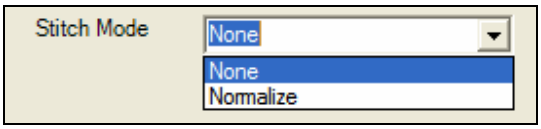
Check the **Add Signatures** check box to enable the signature and audit trail function. Select the **Audit Trail** option box to choose the type of file backup. See detail in the **Security** section.

Stitch Image with Normalization

When stitching two or multiple images to be one image by using **Stich** tools, images can be directly stitch together or all the pixel intensity on the images will be normalized before stitching.

Stitch Mode in

- None: stitch without normalization
- Normalize: normalize all the pixel intensities on each image with the normalization by each slide global mean value of pixel intensities.

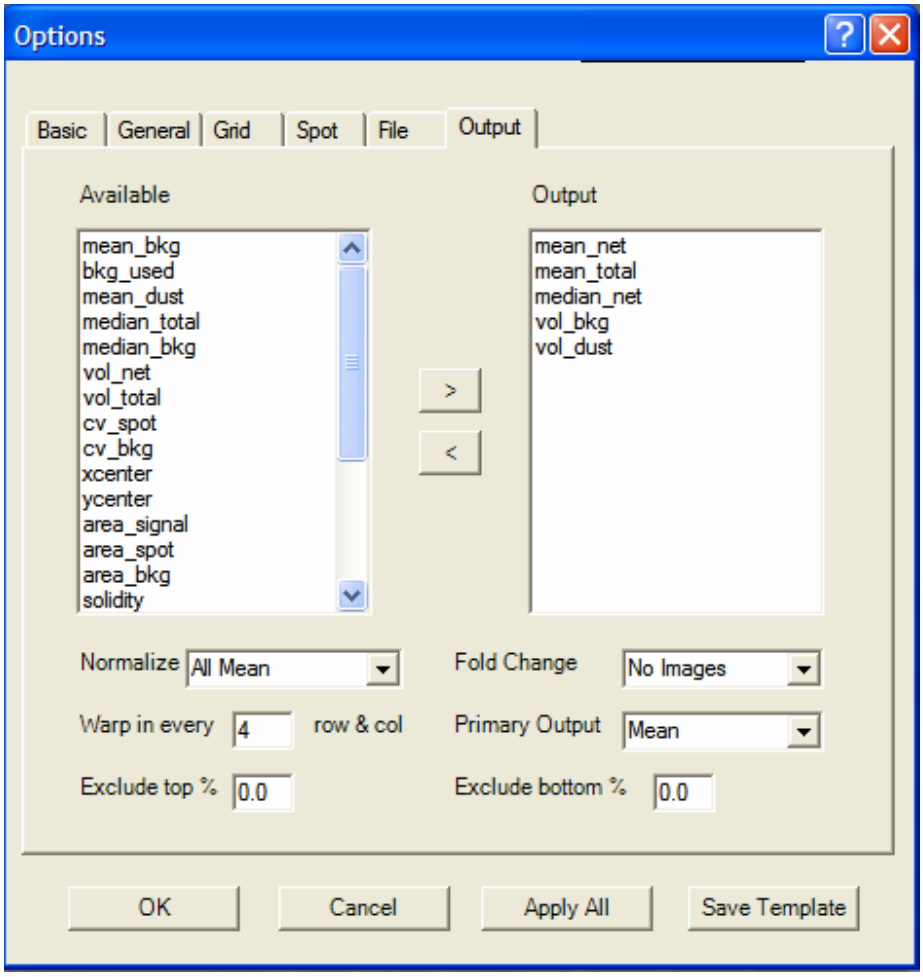


6. Output

The **Output** tab allows specifically desired information to be outputted.

Selecting Output Measurement

The list order in the **Output** text field shows the column order in the output file. Any item can be selected from the list and moved back and forth with the arrow buttons. See detailed information about the measurements in the **Output Data File** section.



Selecting Normalization Type

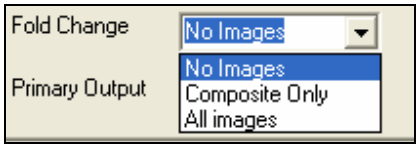
Select a normalization type from the **Normalization** drop-down list to view correlation and fold changes between two images or a composed image. See Normalization in **Review Output Data** section for the details of following normalization types:

All mean|All median|Control mean|Control median|Lowest mean|Lowest median

Selecting Fold Change Option

Selecting Output Spot Image

Select the spot image display or saving option from the **Fold Change** drop down list to display or save individual spot images in the fold change list:



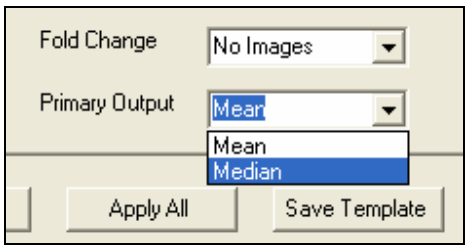
Composite image only:
If a composite image is analyzed by MicroVigene™, it only has a composite spot image.

All images:
Two signal channel images and their composite images. When only two single channel images are analyzed separately, it will show three spot images.

All spot images are cut in squares and the center with the mass center of each of the spots. Later, those spots can be aligned up and overlaid with each other. The spot images are saved in JPEG format.

Define Primary Output

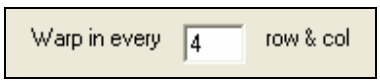
MicroVigene™ currently supports two types of primary output values: **mean_net** or **median_net**. When selecting **Mean** at the **Primary Output** drop down list, it uses **mean_net** to calculate the folds. Otherwise it uses **median_net** for **Median**.



The **Primary Output** intensity value is used for the fold change, correlation scatter plot and **Text & Compare**. Also these intensities will be used with plug-in functions. For example, if the **mean** value is chosen to calculate the **Y0** from the dilution curve, the **Mean** in the **Primary Output** drop down list has to be selected.

Wrapping and Registration Image Overlay Option

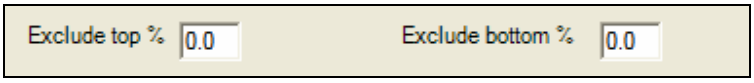
Select the number of rows and columns as registration and wrapping overlay base unit:



For images that are not aligned, such as one in the nylon platform, grid/spots can be found first for each individual image, and then overlaid. MicroVigene™ will automatically warp/register images based on found spots. The number of spots used as control points are determined by the number in the **Warp** field. We recommend setting every 4 rows and columns. Small number yields better registration but may take longer time. MicroVigene™ will use only strong spots as control point to register images if the number is set at **0**.

Trim Spot Signal Pixels for Quantification

For any selected spot intensity measurement, trim top or bottom some percentage pixels in intensity histogram to decrease the variation. This is only happened after the segmentation and spot boundaries are defined.



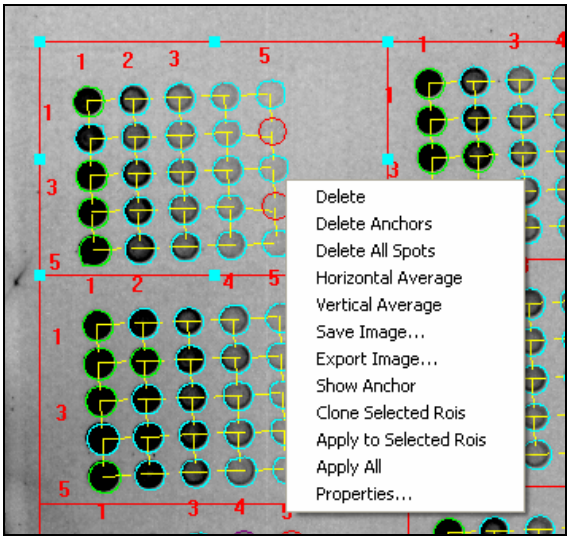
XIV ROI INDIVIDUAL CONFIGURATION

The Configuration or the Option dialog box has the settings for all the ROIs. But each ROI can be set-up individually with a different setting.

1. Setting up ROI Individual Configuration

Opening ROI Pop-up Menu:

Click on toolbar, move the arrow cursor inside of the ROI (not on the spots if image is being analyzed), then left click to select or highlight the ROI, and right mouse click to open the **ROI Pop-up Menu**:



Deleting: Delete grid, spots and ROI frame of this ROI.

Deleting All Anchors: Delete the anchors for selected ROI.

Deleting All Spots: Delete the grid lines and the spot boundaries inside of selected ROI.

Saving Image: Save this ROI array image as a single .tif file.

Exporting Image: Save this ROI array image as a single image file in another format, jpeg, png, tig, gif, or bmp.

If the entire image needs to be exported to another format, just draw a ROI frame over the entire image and then select **Export Image**.

Show Anchor: Manually define the edge of array to be analyzed. See the Manual Tool session

Clone Selected Rois: Create a duplicated set of selected ROIS.

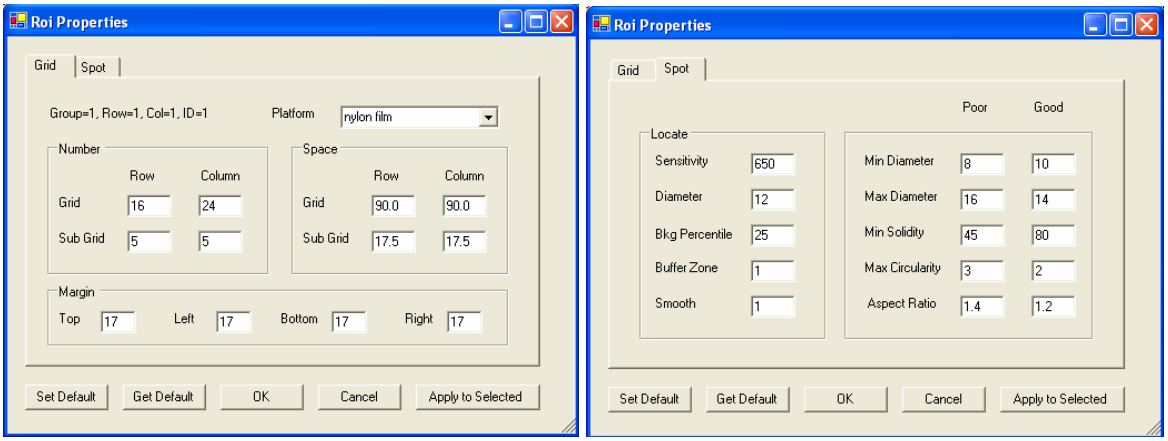
Apply to Selected Rois: Apply current ROI settings to the **properties** setting of all selected ROIs (blue framed).

Apply All: Apply current ROI settings to update the setting in the **Options** dialog box for all default ROIs (red framed).

Properties: Open the **Options** dialog box for the selected (highlighted) ROI.

Configure ROI Individually

Select **Properties** to open the ROI **Properties** dialog box.



The current version of MicroVigene™ supports all the settings shown in the above figure that can be tailored to each individual ROI. Any individually set ROI will have a blue box vs. a default set with a red box.

- **Set Default:** Click to update the default setting in the template file with this ROI setting
- **Get Default:** Load the default setting and over write the current setting
- **OK:** Save the current setting to the template file
- **Cancel:** Cancel current modification
- **Apply to Selected:** Apply current ROI setting to all selected ROI.

XV ID NAME AND CONTROL TYPE FILE - PLATEMAP EDITOR

Tailored data analysis is in the same automatic process if the control information is predefined in the platemap file. Background subtraction with negative control, positive control for normalization reference spots for indexing and excluding blank spots

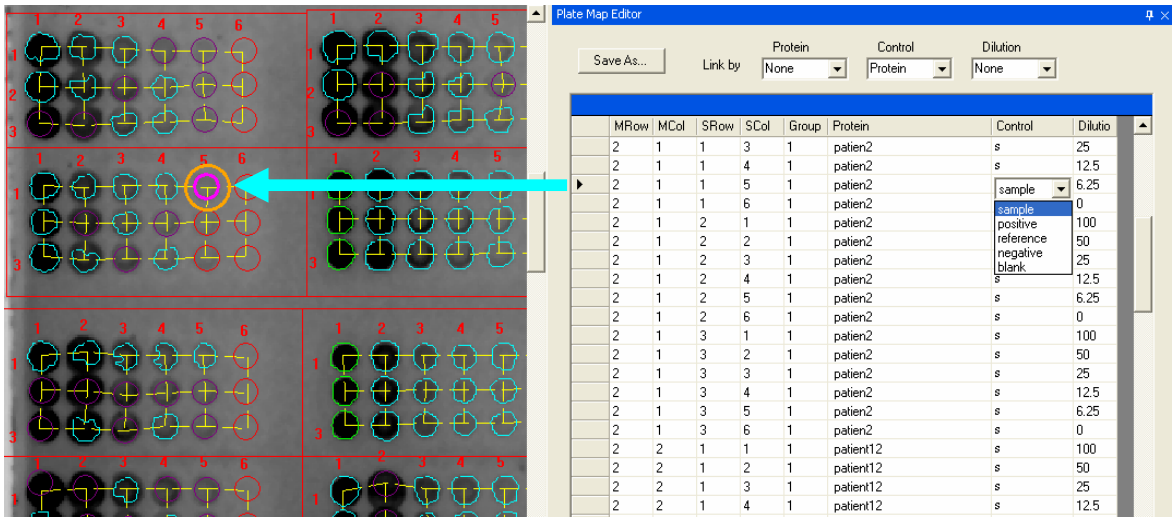
A file with Gene ID file, Protein ID, or sample ID of spots annotation in text format that are generated by scanner or other applications, can be automatically imported to MicroVigene™. An additional column can be added in to support automatic data analysis.

1. Launching Platemap Editor:

After loading the image and selecting the template file or setting up the **Options** dialog box, then analyze the image.

Select **Application::Edit|Platemap** to launch the **Platemap Editor** at the right panel on the screen.

- It will display the imported platemap file. An ID name will fill in the Protein column.
- If there is no entry in the platemap file name field in the **Options::Basic**, MicroVigene™ will automatically list the rows and columns based on the array dimension setting in the configuration and leave the Protein column blank.



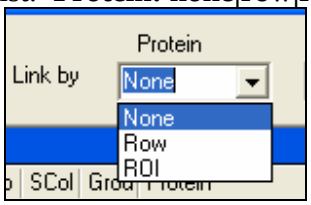
Note: If the image is analyzed, the spots on the image will be associated with the spot rows in the **Platemap Editor** list. Click on the far left gray column to associate the spot row with the spot on the image.

2. Creating and Modifying Platemap

MicroVigene™ has a set of built-in links to simplify the process. For example, if the **ROI** in the **Protein** dropdown list of **Link by** is selected, you click **Enter** once for any row of this ROI, and all the spots will be updated with the same name entry just entered.

Entering ID Name (Gene, Protein or Sample ID)

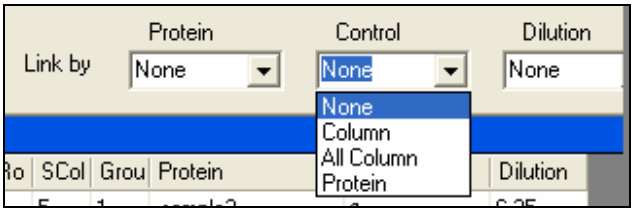
1) If the ID name is the same for the spots in the same row or same ROI, select **Link by** in the drop down list: **Protein: none|row|roi**



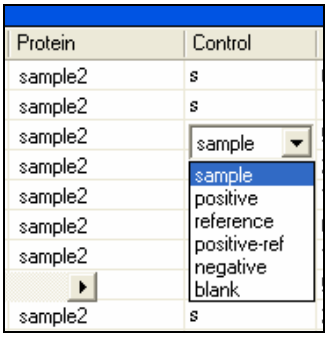
2) Enter or modify the ID name in the **Protein** Column.

Adding Control Type to Platemap file:

1) Select the **Link by** in drop down list for **Control**:**none|Column|All Column|Protein,** if the control type is the same in both column and ID name spots.



2) Click on the **Control** column field for the pop-up drop down list to appear to select the type of control for this spot:



Slide or ROI level normalization or background subtraction:
For multiple slide images, each slide can have its own name, MicroVigene™ can support analysis between slides. It also groups multiple ROIs and does the analysis between each group. Use the Group ID to support the automation.

Creating or Modify Template:
Click **Save As** button after finishing the editor and save it to the platemap file. It will automatically update one in the **Platemap** file field in the **Options::Basic** dialog box.

3. Platemap File Format

The Platemap file is a tab delimitated text file. It can be opened and edited in Microsoft Excel.

protien_Platmap.txt

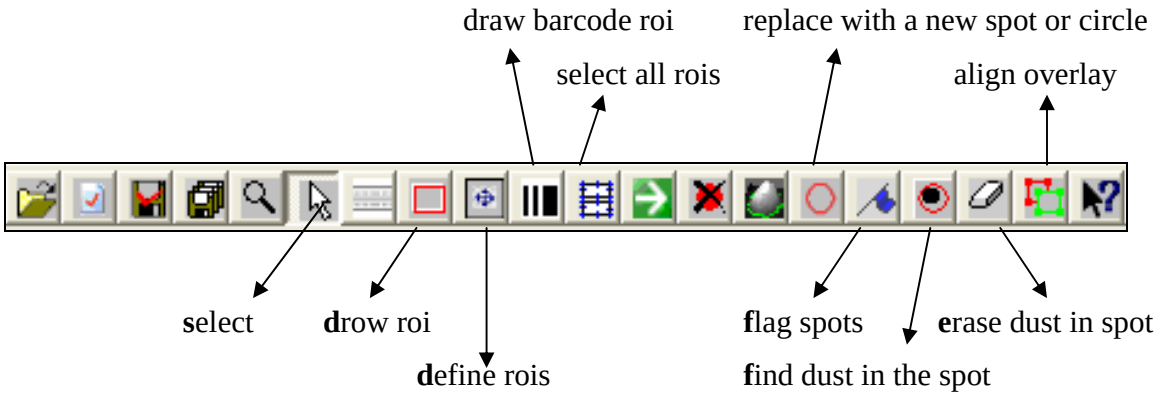
	A	B	C	D	E	F	G	H
1	MicroVieg	Version	1240					
2	Row Count	96	Column C	7				
3	Main Row	Main Col	Sub Row	Sub Col	Gene ID	Control	Dilution	
4	1	1	1	1	Patient1	0	100	
5	1	1	1	2	Patient1	0	50	
6	1	1	1	3	Patient1	0	25	
7	1	1	1	4	Patient1	0	12.5	
8	1	1	1	5	Patient1	0	6.25	
9	1	1	1	6	Patient1	3	0	
10	1	2	1	1	Reference1	1	100	

The control type value in the file is numeral. They are:

Sample:	0
Positive control:	1
Reference standard unit:	2
Positive Reference standard unit:	3
Negative control:	4
Blank:	5

XVI LIST OF MANUAL FEATURES

MicroVigene™ has a set of built-in tools to handle images that fail to be automatically analyzed for the current setting. You can also manually correct or modify the image analysis. Some features can be directly launched from the Toolbar:

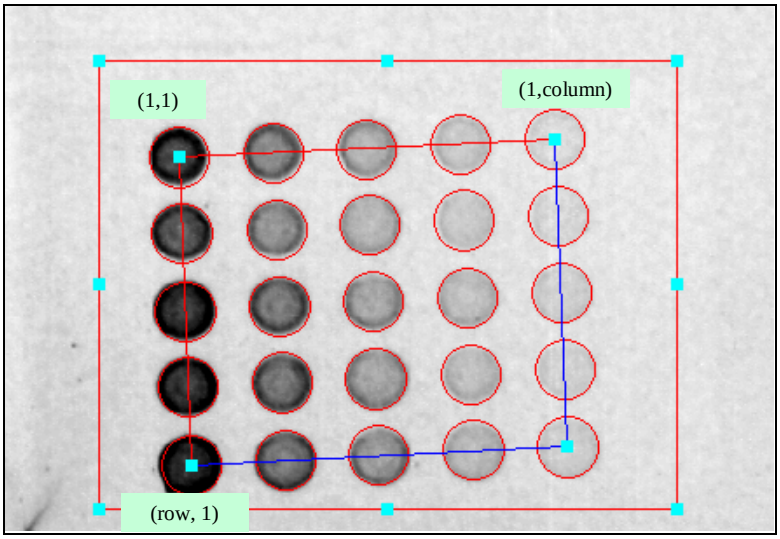


1. Backup Feature

The most powerful algorithm to handle very challenge images cases is the **Show Anchor**. It over rules failed initial automation, and defines the array edge and uses the manually defined array edge to re-analyze the image. In most cases, it can automatically place the grid and find spots correctly. It is a backup function for fully automation process.

Manually Defining Array Edge

Click button on the toolbar and move the cursor inside the ROI. Select or highlight the ROI. Then right click to bring up the **ROI pop-up Menu, and** select the **Show Anchor** in the list to show the anchor frame as below:



There are three anchor points on this frame to define the first row and column of the array.

- Move the top left dot to line up with the center of the first spots (1,1).
- Move the top right dot to line up with the center of the last spots of the first row (1,column)
- Move the bottom left dot to line up with the center of the last spot of the first column (row, 1)

- **Re-analyzing the Image**
  After aligning the anchor frame with the first row and column of the array, the ROI is selected; click button to re-analyze the image.

  Note: The alignment is good for guidance. It does not have to be very accurate. Also, grid is still determined by the algorithm.

  Saving Anchor in Configuration

  The anchor settings can be saved in the template file:

  In the ROI **Properties,** click the **Apply to Selected** button or by selecting the **ROI Pop-up Menu::Apply to Selected ROI** to save.

  Re-analyze the Whole Image with Saved Anchor in Nylon Mode

  - In **nylon** mode

MicroVigene™ will automatically find the grid and spots for all default setting ROIs.

A saved anchor will be used to find the grid and spots for a ROI.

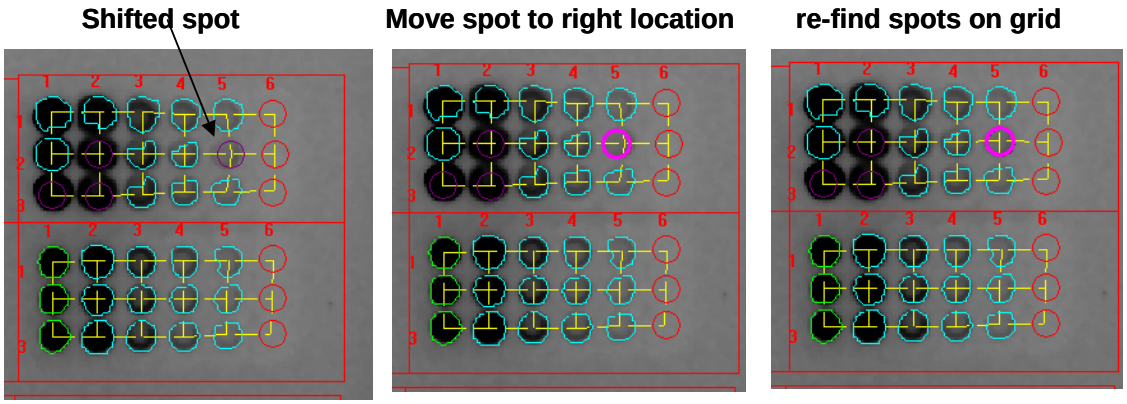
In **Glass** mode,

MicroVigene™ will automatically find grid and spots for all ROIs. For a ROI with a saved anchor, it will have to be analyzed when the ROI is selected.

2. Modifying and Improving the Grid and Spots Finding

If you find that a grid line is shifted from the right location, you could fix it quickly by manually moving the spot to the new position as a new anchor point. Then MicroVigene™ will automatically re-define the grid and spots along with the grid line.

Click on the Toolbar, then click and move the shifted spot to the right position. Then select the **Application Menu:: Analysis|Re-find spots on the grid** or the **F3** function key**.**



3. Modifying and Improving Spot Finding

If there are a few spots that are missed by the current setting and the grid is still fine, you could fix it quickly by manually moving the spot to the right position. Then MicroVigene™ will take the manually adjusted position to do the quantification for this spot.

Click on the Toolbar, then click and move the shifted spot to the right position. Then select **Application Menu:: Analysis|Calculate Signal** to update all the spots intensity after the spots are moved..

4. **Manually Flagging Spots and Removing Dust:**

   In the Spot Data Analysis section, it will have more detailed information.

5. **Manually Defining ROI for Irregular Array or Auto Define ROI Failure**

   For irregular layout array, due to some quality of the array, there is not enough reliable information to automatically define a ROI., a tool may let you manually draw each ROI frame.

   Click on the Toolbar, move the cross cursor to the image. Click and drag the ROI red frame out side of the ROI.

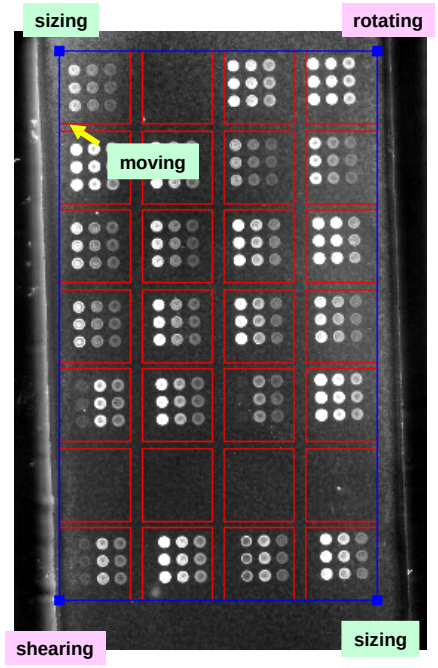
   Note: Based on the configuration setting in the **Options::Basic** dialog box, the total number of the combination of **ROI Row** and **ROI Column** defines how many ROI can be drawn on the image.

6. **Manually Creating and Adjusting ROI frame**

   This feature handles the failures of automatic defining ROIs in any array.

   Click on the Toolbar, to lay an ROI frame on top of image based on array and grid setting in the template file. Four corners server as control points to adjust the frame.

   Single Slide Image:



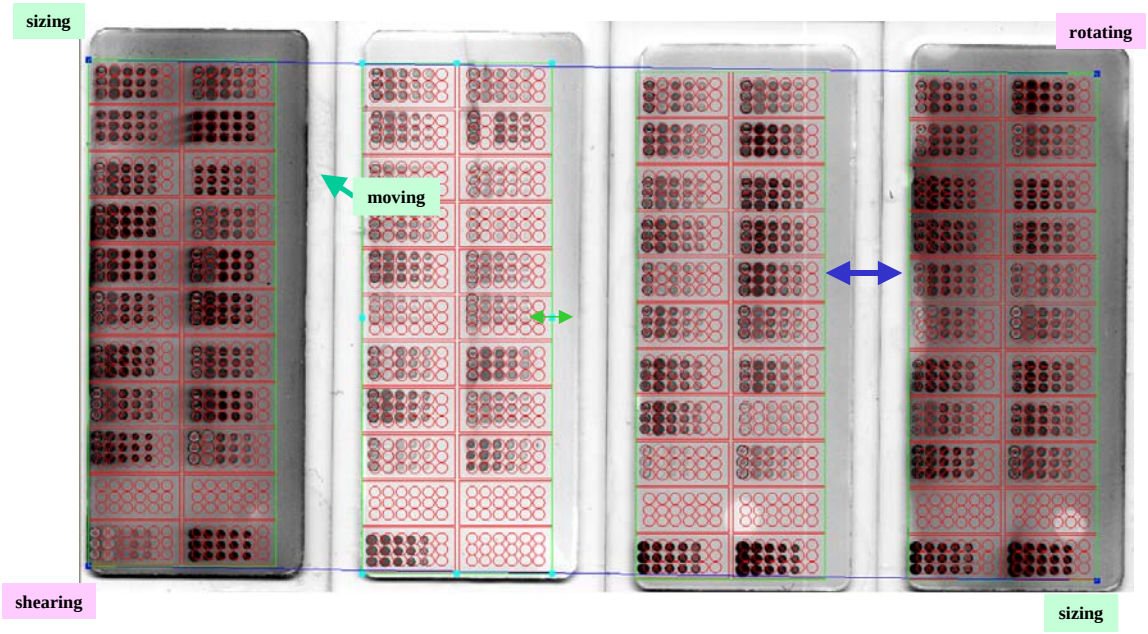
As shown above, click and move on a blue squared corner of the blue contour to **rotate**, **shear**, and/or **size** the ROI frame to lay right on top of the array grid. To move the whole blue and RED ROI frame, move the cursor inside the blue frame, but in the gap between the ROIs, then click and drag the whole frame globally.

Note: Do not move the blue frame to the side edges of the images. When the blue frame is over the edge of image, the ROI box will be misplaced and will have to start over again.

Enlarging Blue Frame with Enlarge the Spots Spacing
Click and drag the top left blue square or the bottom-right square to enlarge or decrease the size of the blue frame. The grid spacing and ROI size will increase or decrease proportionally with the blue slide frame.

Enlarging Blue Frame with Fixed Spots Spacing:
Hold down the **Shift** key, then click and drag the top-left blue square or the bottom-right blue square to increase the gap between the ROIs and keep the ROI size and grid spacing the same when enlarging the blue frame.

Multi-slides Image



To move the blue multiple-slides frame, move cursor between the slides, hold down the left mouse button, and move the cursor to move the entire blue frame.

Enlarging Blue Frame with Enlarge the Spacing between spots, ROIs and Slides:
In addition to single slide images, click and drag the corner square. The slide's spacing will also be changed.

Enlarging Blue Frame with Fixed Spacing between Spots, ROIs and Slides:
Hold down the **Shift** key, then click and drag the blue corner square. Only the space slide will be changed.

Adjusting Green Slide Frame Individually:
Click the inside of a green slide frame to select the slide. Adjust the side squares for changing the slide size, ROI, and spot spacing the same way as working with a single slide image.

Note: The four corner green squares are not the anchor points to adjust in this version.

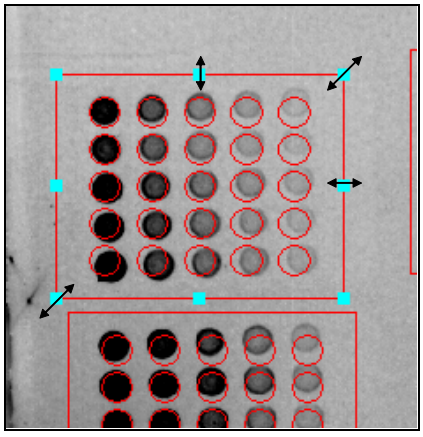
7. Manually Adjusting ROI Frame and Configure Grid

Adjusting ROI Frame

Click on the Toolbar, and then click inside the ROI to select a ROI.

Moving ROI frame Globally:
Move the cursor inside of the ROI frame, hold down the left mouse button and drag to move the whole ROI frame on top of the array area.

Adjusting ROI Size
Click on the cyan squares on the ROI frame to change ROI side individually, as shown below.



Enlarging ROI Frame with Enlarged Spots Spacing:
Click and drag a corner cyan square to change the size of ROI frame in two dimensions and a site square in one dimension. The spot spacing in the rows and columns will be proportionally changed with the ROI frame.

Enlarging ROI Frame with Fixed Spots Spacing:
Hold down the **Shift** key, then click and drag a cyan square to keep the spots' spacing the same as when changing the ROI size in one or two dimensions.

Note: If the Anchor is set, the up and left margin will not change when holding Shift to change the ROI size. If changing the margin is needed, you'll have to delete the Anchor first..

Configuring ROI with Graphic Dimension

After aligning the circles with spots on the image, you can save current grid and ROI layouts in the configuration or the **Options** setting or the ROI **Properties** setting by:

Updating to Options (for all ROIs)
Right click on the selected ROI, then select the **ROI Pop-up Menu::Apply All** from the menu list.

Updating only to selected ROI

Hold down the Ctrl key to select ROIs. Move the select cursor to the aligned ROI, then right click to select the **ROI Pop-up Menu::Apply to Selected Rois**

XVII CONFIGURATION SET UP STEPS

1. Regular Image

There are several configuration tutorials in MicroVigene™, On-line Help. This is a list of general steps.

1. Click the button on the Toolbar and use the folder browser to open a good image file.

2. Visually exam the ROI matrix, how many rows and columns are in the image and how many rows and columns of spots are in each ROI. This information may also be obtained from its gene ID or platemap file.

3. Click the **Select** button on the Toolbar to check the gray level of spot's area and background area. Verify if it is a **positive** or a **negative** spot image.

4. Use the **measure length** button on the Toolbar to take 5 measurements, ROI gap and spots spacing in vertical and horizontal, and the spot diameter. If there is some high background in the surrounding area outside the array, measure the cut off margins in pixels.

5. Click the **options** button on the Toolbar to open the **Options** dialog box to set up the configuration.

   In the **Options::General** tab type a new template name in the **Options::General|Template** name box, click the **Save Template** button to create a new template.

Delete any file names in the **Options::General|Plate Map** name box during the first tuning.

In the **Options::Basic** box:

In the **Number** section, update the **ROI** and **Grid** fields of **Rows** and **Columns.** Set the **Sub Grid** fields the same as **Grid** if it is for **glass** mode.

Set the **Sensitivity** at 850 or 900**.**

Enter the average spot's **Diameter**

In the **Options::Grid** box

- In the **Space** section, update the **ROI** and **Grid** fields in **Rows** and **Columns.** Set the **Sub Grid** fields the same as the **Grid** if it is for **glass** mode.
- In the **Margin** section, set up the cut off margins as needed**.**
- The typical setting of Max **Shift (pixel)** is 1/3 of the Grid spacing. It varies upon level of pin displacement.

In the **Options::Spots** box

- Update the check box of the **Positive Spot** with the information in Step 3.

Click the **Save Template** again.

6. Check the configuration of the grid and spots.

1) Click on the Toolbar, move the cursor to the image, then click and drag to make the ROI frame clearly around a ROI.

2) Click on the Toolbar and move the cursor inside the ROI, then click to select (highlight) the ROI

3) Click on the Toolbar to analyze one ROI.

4) Check the grid placement and spot finding. If it is correct, this means the setting for finding spots and configuration setting for spots are also correct.

7. Check Configuration of ROI

1) Click button on Toolbar to lay ROI frame on top of the image. Click inside the Blue frame and drag whole ROI frame to place top of the array. Check the dimension of ROI frame to see if it fits whole array image.
2) If this ROI frame does not suit the array image, graphically and manually adjust ROI size and update with selected ROI setting to all ROIs. See **Instruction** in **Manually Configure ROI** section. Then select all ROI , click to run again.
3) If successful, delete ROI frame by selecting **Application Menu::Edit|Delete All Object**
4) Check ROI **relocate** mode at **Options::General** box. Then click **Application Menu::Analysis|Find|ROIs** only to automatically defined the ROIs.
5) If the ROI frames are correctly placed at the top of the array image. Then use **Application Menu::Analysis|Find|Grid and Spots** to find all the image spots.

8. Test with one mouse click

Delete all the grid and spots, **Application Menu::Edit|Delete All Object.**

9. Click to test the one mouse click operation.

10. Save configuration to the template file by clicking .

2. Configuration for Challenged Images

There are rules for adjusting configuration setting to handle challenged images. In the Options dialog box:

If you have:	You need to:
Weak image ---	Increase **Sensitivity**
Noisy image ---	Decrease **Sensitivity**, Set **Margin**s
Shifted image ----	Increase **Max Shift(pixel)**
Dusty image ---	Decrease **Threshold** and **Dust mean**
Irregular spot shape image ---	Increase **Smooth**
Less than good strong spot image ---	Increase the range for **Good** spots definition
Dusty image ---	Decrease **Threshold** and **Dust mean**